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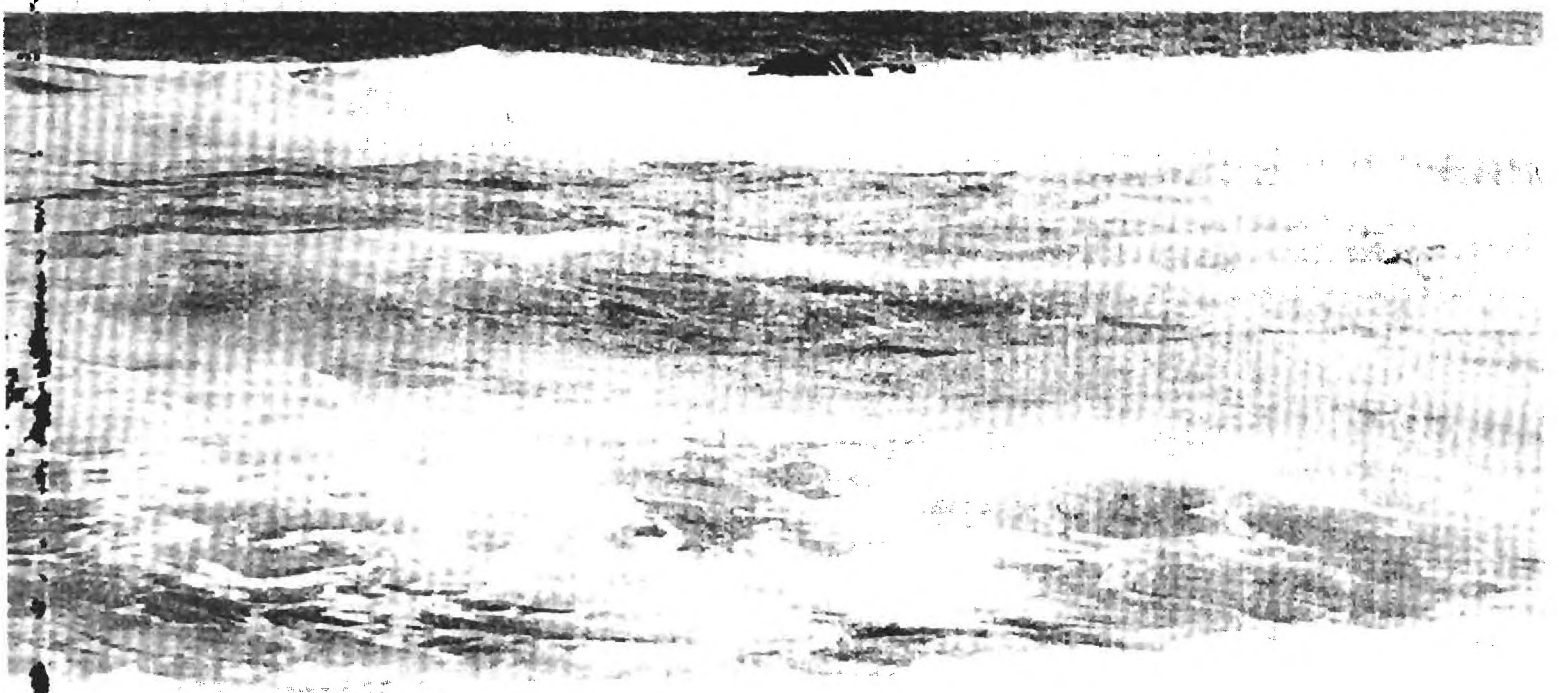
ERC-1076

October 1976

EFFECTS OF HIGH LEVELS OF INORGANIC PHOSPHATE ON AQUATIC ORGANISMS IN PHOSPHATE-RICH ENVIRONMENTS

by

John R. Strange



School Of Biology

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GEORGIA INSTITUTE OF TECHNOLOGY
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Effects of High Levels of Inorganic Phosphate on Aquatic Organisms in Phosphate-Rich Environments

By

John R. Strange

Project Completion Report

OWRT Project No. B-088-GA

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School of Biology

in cooperation with

Environmental Resources Center

Georgia Institute of Technology

Atlanta, Georgia 30332

AUTHOR

Dr. John R. Strange was an Assistant Professor in the School of Biology at the Georgia Institute of Technology until June 30, 1976. He is currently employed as a Senior Scientist at the Syracuse Research Corporation in Syracuse, New York. He served as the principal investigator for the project which is the subject of this report.

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ledge of the problems associated with the eutrophication of a large recreational waterway. Their time and help was invaluable and each one played a unique role for which I am most grateful.

ABSTRACT

During the course of this three-year study (1973-1976) on Lake Lanier, the water quality in the Flat Creek Embayment did not change appreciably. Water quality is somewhat better than it was five years ago. Bacteriological evaluation of the Flat Creek water revealed larger numbers of coliforms present in 1975 than in 1973.

A process for monitoring cardiac and opercular rates in the channel catfish was developed utilizing unimplanted electrodes. Cardiac rates increased with increasing phosphate levels up to 15-20 mg/l. No further increases were observed at levels up to 160 mg/l. Chronic exposure at 20 mg/l phosphate revealed no accomodation to the phosphate-induced cardiac rate elevation.

A technique developed for monitoring basal metabolic function demonstrated increased metabolic rates in the channel catfish with increasing phosphate levels up to concentrations of 10 to 15 mg/l.

In a food conversion efficiency study, catfish exposed to 10 mg/l phosphate grew faster than controls. Fish exposed to 20 mg/l fell between the controls and the 10 mg/l group. Although the phosphate-exposed fish grew faster and ate more, their food conversion efficiencies were similar to controls.

Biochemical studies in which the activities of the enzymes lactate dehydrogenase (LDH), pyruvate kinase (PyK), and hexokinase (HK) were monitored after exposure to phosphate and sulfate gave varied results. It was suggested that phosphate increases the activities of LDH and HK while decreasing the activity of PyK. Sulfate was determined to exert no effect on these enzyme systems.

Attempts at electrophoretic separation of the LDH isozymes met with moderate success. Two major and two minor bands were found to be present using cellulose acetate strips. Starch gel separation proved inadequate.

Direct labelling with P-32 phosphate showed incorporation of phosphate in Daphnia and channel catfish. Peak activity was detected in the Daphnia by 2 hours of exposure with no further increases up to 48 hours exposure. Most catfish tissues continued to incorporate additional phosphate throughout the 2-week test period. Catfish fed exclusively P-32 labelled Daphnia showed different phosphate incorporation ratios than those fish exposed directly to the radiolabel. The liver tended to sequester proportionally larger amounts of P-32 in the Daphnia fed experiment than in the direct exposure experiment.

Keywords. Eutrophication, Channel catfish, Daphnia, Phosphate, Cardiac Rate, Metabolic Aberrations, Lake Lanier Water Analysis.

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CHAPTER 1. INTRODUCTION

In an effort to better his existence, man's exploitation of easily available resources has brought about some unexpected and undesirable results. With little attention to conservation or efficiency, man has caused a depletion of once considered inexhaustible resources and a contamination with wastes of the remaining ones. To maximize return and minimize costs, domestic and industrial wastes have been deposited in the nearest waterway as a means of disposal. As the United States population continues to grow over the two hundred million mark, aquatic ecosystems are no longer able to assimilate the volume of wastes to which they are being subjected. This problem is further aggravated by the need to feed this growing population, which requires a highly productive agricultural system. Unfortunately, in maintaining a productive soil monoculture, heavy fertilization is necessary. Runoff from fertile land makes fertile water which is also productive. This nutrient enrichment of the water gives rise to algae blooms and other undesirable biotic changes.

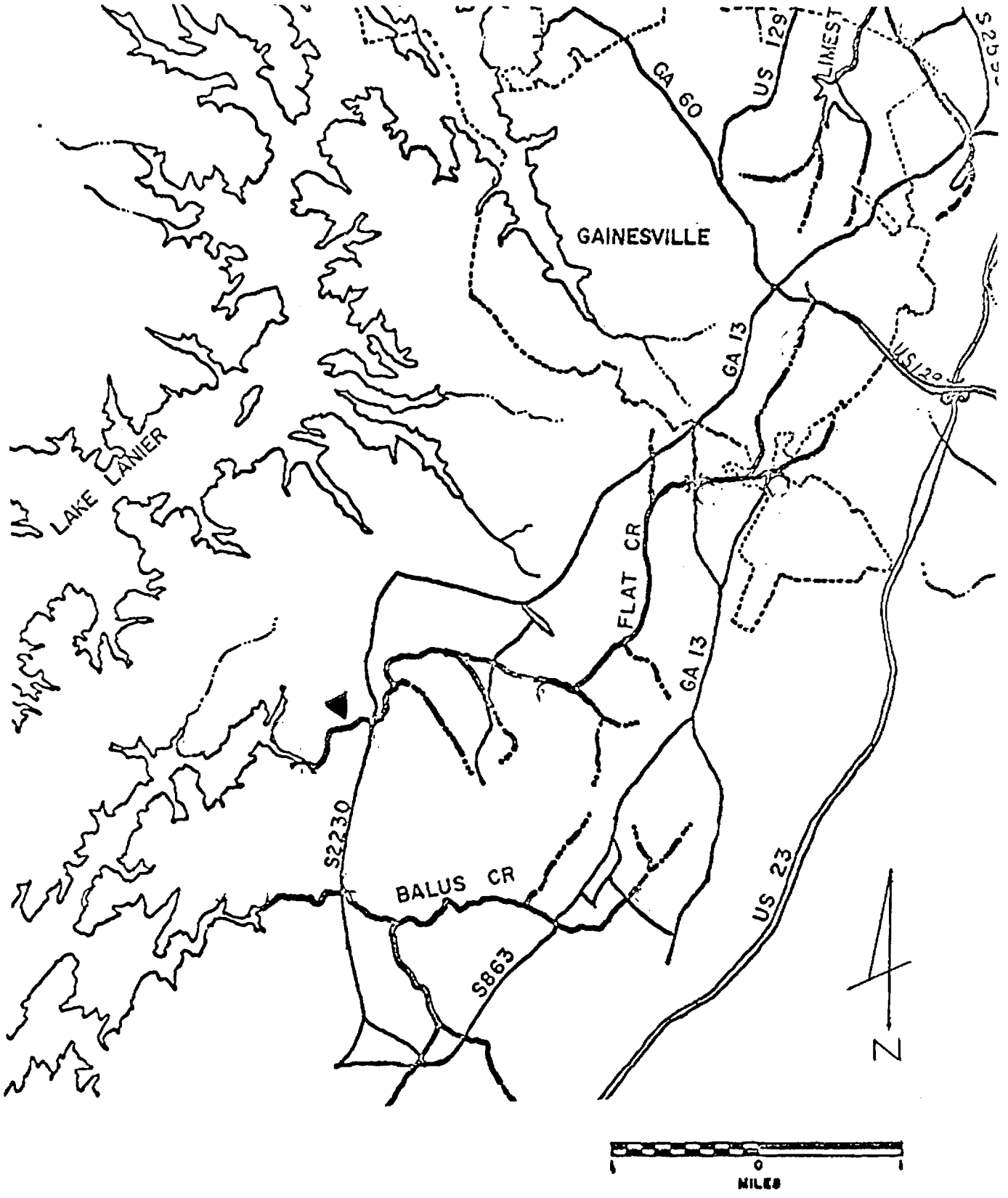
The Flat Creek Embayment of Lake Sidney Lanier has undergone nutrient enrichment or eutrophication of this sort. Lake Lanier is formed by Buford Dam, an earthen dam completed in 1957. Located northeast of Atlanta, Georgia and just west of Gainesville, Georgia, Lake Lanier is a multi-purpose project with the following officially recognized functions: (1) Power - hydroelectrically produced energy; (2) Flood Control - flood control downstream on the Chattahoochee River; (3) Navigation - increased flow for the Apalachicola River during low-flow periods.

Recreation is not one of the official project purposes; however, in 1973 Lake Lanier recorded almost 14 million recreation days to make it the most heavily used Corps of Engineers lake in the United States (1).

The Flat Creek embayment (see Figure 1 for location) of Lake Lanier is in close proximity to the industrialized south side of Gainesville. Upstream from this embayment, numerous outfalls from various sources have at one time or another contributed to the poor water quality of this section of the lake. The Georgia Quality Control Board, the District Department of Environmental Sanitation, the City of Gainesville, and the Hall County Health Department have cooperated with the companies involved to remove or control the sewage and industrial outfalls that empty into Flat Creek. This has improved the situation, as will be discussed later in this report, but problems still exist. Details of this situation are given in Appendix III of the OWRR Project Number A-035-GA completion report by J. R. Strange (2).

In the eutrophic conditions found at Flat Creek, the nutrient loading is so heavy that the self purification ability normally found in a natural aquatic ecosystem is absent. When production in a system is approximately equal to respiration, then the water will remain in a nonpolluted, aesthetically pleasing state. In this balanced state, oxygen gas and organic matter are produced by photosynthesis. The oxygen produced is then used by heterotropic organisms that decompose the organic material produced. When a temporal or spatial separation occurs between production and respiration, such as that found at Flat Creek, then chemical and biological changes take place.

Figure I



▲ Sampling site

If production is much greater than respiration, it is usually manifest by an accumulation of algae. When respiration is much greater than production, dissolved oxygen is depleted and NO_3^-, SO_4^{-2}, and CO_2 may be reduced to N_2, NH_4^+, HS^-, and CH_4.

In the embayment at Flat Creek, a physical separation of productivity and respiration has taken place. The algae, or producers, are found only in the euphotic upper zones. The O_2 is evolved to the atmosphere as photosynthesis takes place, whereas, decomposing algae and other detritus settle to the lower layers of the lake. Here, there is no more oxygen production or photosynthesis and with the rapid accumulation of organic matter on the bottom, the dissolved oxygen in the water is rapidly depleted. This results in a heavy sediment with a high biochemical oxygen demand. If oxygen is not available to the heterotrophic organisms on the bottom, a gradual filling of the lake with sediment will occur.

At Flat Creek, a common cyclic pattern has occurred over the past few years. During the summer, fall, and winter months, production has taken place as described above and an accumulation of nutrients has occurred in the lower strata of the lake. With the warming trend that takes place in the spring, a mixing or overturn of the waters in the lake occurs. An increase in the concentration of available nutrients in the water results, and the sudden availability of nutrients along with increased periods of sunlight has given rise to algae blooms in recent years (3, 4). These blooms have caused several fish kills as the dense algal mats have prevented gas exchange necessary to maintain sufficient oxygen content in the water.

The nutrients causing the algal blooms in Flat Creek come from the remaining industrial and domestic outfalls that have not been eradicated and the leaching of nutrients complexed with the bottom sediment.

A variety of conditions must exist in the embayment area for optimal productivity or algae growth to take place. Principal elements such as C, N, P, K, Mg, and S are needed for synthesis by aquatic organisms. Several multivalent ions and organic growth factors such as vitamins and hormones may also be required for optimal growth. Carbon in the form of CO_2 or carbonate (HCO_3^-) is readily available in natural waters as are most of the other trace elements. The nitrogen and phosphorus concentrations in inland waters are usually the growth limiting factors. A nitrogen deficiency is easily remedied by any of the blue-green algae species capable of assimilating and fixing nitrogen, therefore, in most circumstances, phosphorus is the influencing factor on productivity.

Phosphate levels are more often altered by man's activities than any of the other elements in water pollution. Input of phosphorus into a system of course depends upon a variety of factors. At the Flat Creek Embayment, the major considerations seem to be industrial runoffs and sewage effluents. With the city of Gainesville in the Flat Creek drainage basin, cultivation is very minimal in this area and not a significant source of nutrient loading. The problem is not alleviated to any great extent by common aerobic sewage treatment practices, which are capable of removing less than 50% of the phosphate components in domestic sewage. The major source of the phosphates in domestic sewage in recent years has been the salts of various polyphosphoric acids added to detergents as water softening

agents. In a septic environment, it has been demonstrated that the polymers of orthophosphate are converted to orthophosphate, which is the form that aquatic plants utilize (5). More effective removal of phosphates can be realized in a variety of ways. Addition of iron, lime, or aluminum salts, or altering pH results in chemical precipitation of high concentrations of phosphate. Jorgensen et al have reported that these methods are much more effective if a high concentration of suspended matter is present to assist in complexing the phosphate ions (6).

Kalesy has examined the role that algae play in purification of sewage waters and has found that the removal of nitrogen from effluents is proportional to the dry weight of the algae. Phosphorus removal by algae was much more effective, but was not found to be correlated with algae weight. Physicochemical properties of the environment were also found to alter the water purification capabilities of algae (7).

Greer and Ziebel also utilized algae in an eutrophic water purification study. After algae had incorporated the excess phosphorus, clam beds were used to remove the algae (8). Peterson et al have also attempted using aquatic plants to remove excess nutrients from water, but found that even with full scale harvesting of these plants only 1.37% of the phosphorus deposited in a suburban lake could be removed (9).

Current methods for removal of phosphorus from municipal treatment plants are reviewed in an article by Jones (10).

Even if all of the phosphates were removed from sewage effluents, less controllable non-point sources would still be a major problem. It has been estimated that removal of 90% of the phosphates in waste water would

result in a reduction of only 40-80% of the total found in a lake (11). This seems like a trifling amount, however, a reduction of this order during certain periods of the year could help reduce or do away with algae blooms such as the ones found at Flat Creek.

Technological advances in water treatment have progressed sufficiently in recent years to avert large scale crisis situations, but the causes of many problems remain. The solutions, although not always economical, are available. Man will continue trying to realize maximum profit on his investments until he is forced to draw a compromise between "how much" he can acquire and the quality of what it is he wishes to acquire. Industries and private businesses guilty of polluting waterways have been cooperative with officials of environmental agencies, but have been slow to initiate water improvement programs on their own.

With more complete control of pollution problems several years away, it is vital that further insight into the fundamental biological and chemical functions of both undisturbed and stressed ecosystems be gained. Such information will be useful in the future to (1) recognize existing pollution problems, (2) establish guidelines by which to alleviate such problems, and (3) be able to predict changes that may be brought about in a given ecosystem.

The principal investigator of the current project has had previous experience with the phosphorus problem at Flat Creek. OWRR project number A-035-GA examined the utilization of phosphorus by selected phytoplankton species endemic to the Flat Creek Embayment, and it was found that these organisms would grow at an accelerated rate with the availability of luxury

amounts of phosphorus. An inverse relation between surface area of the algae and radiolabelled phosphorus uptake was also suggested. Similar results have been obtained by other investigators (12). Keenan and Auer (13) considered the effects of starving the algae prior to measuring uptake, a situation similar to the algae grown in phosphate-free media by Strange (2). The suggestion that phosphorus and nitrogen is the limiting growth factor for algae in fresh water is further supported by the work of Schelske and Rothman (14). Condit, working in a selected river flowing through a suburban area, has also noted similar properties of phosphorus utilization by algae. He demonstrated that algae bio-mass production increased as phosphate concentrations increased (15). Finenko and Krupatkina-Akinina have examined the effect of phosphorus on diatom growth rates and have reported an increased in cell division with increased concentrations of phosphorus (16). Similar results were reported in Fragillaria sp by Strange (17).

Experimental evidence such as the above indicates that phosphorus does indeed affect the functioning of an ecosystem at the level of primary production. The purpose of this project is to go beyond a single trophic level and examine the flow of phosphorus throughout the entire ecosystem. Efforts have been made to determine what chemical and biological effects high levels of phosphorus have at a system level. Particular attention has been given to metabolic effects and physiological changes that occurred in the experimental organisms.

Chemical Monitoring

A close check on the chemical pollutants that have been introduced into the Flat Creek Embayment from the headwaters of Flat Creek was

maintained throughout the course of this project. In addition to the data obtained during this three year period, two previous years' data are available to the investigator from OWRR project number A-035-GA. Monthly samples have been taken for the five year period to give yearly means for various water quality parameters. Additionally, two 48-hour analyses of the creek were conducted with hourly sampling. This was done in order to examine fluctuations of the nutrient loading on a diurnal basis.

Bacteriological monitoring was also conducted during the three years and the resulting data were examined along with the chemical data to assess the overall quality of the lake water. From this information, the results of efforts to remove problem sources of pollution within Flat Creek have been evaluated.

Biological Monitoring

With the utilization of phosphorus by primary producers already examined in some detail, it was beneficial to study organisms of higher trophic levels in this ecosystem. Two selected heterotrophic organisms that rounded out the food web in a representative manner were the zooplankter Daphnia sp. and the channel catfish, Ictalurus punctatus.

The organisms were examined indivudually and in interaction with each other. The Daphnia were used primarily to gain knowledge of the flux of phosphorus through an aquatic ecosystem while the catfish were examined more closely for metabolic aberrations. Studies were undertaken to determine to what extent Daphnia incorporated phosphate and what extent bioaccumulation and biomagnification of phosphate occurred. To study further the

phenomenon of bioaccumulation, these Daphnia were fed to catfish fingerlings. Bioaccumulation in an organism can have additive, synergistic or even toxic effects when threshold levels are reached. The channel catfish is at the highest trophic level in the Flat Creek area and should represent the ultimate in bioaccumulation of phosphate if it occurs. It should also be remembered that these fish can serve as a food fish for man and that the possibility of bioaccumulation and monitoring of the same should be watched closely.

In an attempt to monitor some of these parameters, a new method of measuring opercular and cardiac rates was developed. Methodologies employed by Strange et al, to obtain both opercular movement and an electrocardiogram trace have not previously appeared in the literature (18).

Cairns and his coworkers have examined in some detail the use of fish in water quality monitoring. According to Cairns, such use of fish as biological monitors will (1) give warning of potentially hazardous conditions in time for specialists to be called in to evaluate the problem, (2) perceive toxic conditions that pass physical sensors undetected, and (3) give continuous, immediate information on the toxicity of the effluent to aquatic organisms (19). The bluegill sunfish (Lepomis macrochirus Rafinesque), a warm water specie of fish, has been recommended by the U.S. Environmental Protection Agency for use in bioassay studies. Cairns has used this particular fish and the Flagfish (Jordanella floridae) in the detection of toxic levels of detergents, insecticides, and zinc (20, 21, 22, 23, 24, 25, 26). One of the methods used to measure toxic effects of

these chemicals has been the respiratory rate of the bluegill (22, 24, 25, 26). In measuring the respiratory rates, Cairns employed either implanted cannulas or electrodes. Fish swimming and movement patterns have also proven to be reliable indicators of the presence of toxic chemicals (22, 25, 26, 27).

In the method developed by Strange et al, both respiratory and cardiac rates were obtained without the use of implanted electrodes. This procedure greatly reduces the trauma associated with implantation and maintenance of electrodes.

This procedure differs from a similar design of Rommel (28) in that his procedure was in salt water and the water resistance at which the best readings were obtained was 1000 ohm/cm, whereas, the procedure of Strange et al utilized fresh water with a resistivity of 4,600 ohm/cm. Also, two separate channels were used, thus allowing complete separation of cardiac and opercular rhythms.

With a method developed to measure cardiovascular function available, a corollary measure of metabolism was pursued. This phase examined how the metabolic rate or consumption of O_2 varied in the catfish with varying levels of phosphorus.

Information concerning food consumption and utilization efficiencies in the catfish is incomplete. Studies examining the influence of temperature and photoperiod length upon growth are available (29), but work concerning the effects of phosphorus is scarce. For this reason, the catfish were closely studied to correlate food consumption with growth.

Biochemical Monitoring

Lee, in his article concerning the role of phosphorus in eutrophication (30), has suggested that enzymatic studies of organisms subjected to high levels of pollutants be conducted. This type of data would be

useful in any biological evaluation of a pollution problem. Selected enzymes in the catfish were examined spectrophotometrically and electrophoretically to determine if they were altered by phosphorus.

The enzymes that were examined in this project are all important in intermediary metabolism. These metabolic pathways are composed of a series of degradative and synthetic steps that are facilitated by enzymes. The enzymes lactate dehydrogenase (LDH), pyruvate kinase (PyK), and hexokinase (HK) are all important in metabolism and are part of a complex and intricate set of checks and balances that regulate the functioning of an organism's metabolism. With such interaction, small changes in one of these metabolic enzymes would be characteristic of changes in the metabolic pathways of the fish.

LDH catalyzes the terminal step in glycolysis in which pyruvate is reduced to lactate. The LDH enzymes that carry out these reactions are abundant and relatively easy to evaluate experimentally. This enzyme is present in vertebrate muscle and consists of two different type subunits which combine in groups of four. The two types of subunits are referred to as heart (H) and muscle (M) subunits which can combine in any of the five possible permutations. H subunits are characteristically found in muscle that depends upon a readily available oxygen source. An example of such a muscle is the heart. M subunits usually are found more in skeletal muscle which is better able to function in an anerobic state for short periods of time. Stresses placed upon an organism may alter the composition or electrophoretic mobility of these isoenzymes. LDH, therefore, affords a measure of the effects of stress on catfish metabolism. Additional reasons

for selecting LDH are: (1) changes in LDH patterns are symptomatic of cardiac anomalies, and (2) the LDH isozyme system has not been adequately described in the catfish.

The other two enzymes considered in this study are regulatory enzymes that serve to control the supply of substrate required for metabolism. Pyruvate kinase catalyzes the production of pyruvate from phosphoenolpyruvate (PEP) by a transphosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). This is a rate limiting enzyme at an important branch and control point in gluconeogenesis and glycolysis. The activity of the enzyme is affected by the buildup of the products of the reaction. As the product molecules (pyruvate) build to higher levels than the cell requires, the equilibrium of the reaction shifts in the opposite direction and PEP and ADP may be produced. Monitoring the activity of this enzyme will detect metabolic changes.

Hexokinase phosphorylates glucose at the number six carbon position. This provides an alternative to glycogenolysis and results in an increased supply of substrate to the glycolytic pathway. As with pyruvate kinase, hexokinase activity is regulated by levels of metabolites. In this case the metabolites are glucose-6-phosphate, ADP and ATP. Again, changes in this enzyme system are indicative of changes in the entire metabolic system.

Phosphorus Flux

A series of experiments were conducted to approximate the flux of phosphorus in the Flat Creek ecosystem. A previous study by this investigator (17) laid the groundwork from which the problem was approached. Methods similar to the ones used in this experiment with P-32 uptake in lentic algae

were employed to conduct uptake studies in both Daphnia and catfish. Emphasis was then shifted to a holistic approach and a microecosystem was established in which P-32 labelled Daphnia were fed to unlabelled catfish. When all of this information was pooled, somewhat of a quantification of the phosphorus flow in the entire system was afforded.

Project Objectives

The fundamental purpose of this project was to gain information concerning the role of phosphorus in eutrophication. Continuing investigations into the problems caused by eutrophication will result in an increased ability to manage and control the future of freshwater resources.

Specific purposes of the project were:

1. Monitor the water quality of Flat Creek - relating in-field phosphate concentrations to in-laboratory experimentation.
2. Conduct an in-depth investigation into the effects of phosphorus on cardiac and respiratory function in the catfish.
3. Determine what effects high levels of phosphorus have on metabolic and biochemical parameters in the catfish.
4. Measure the incorporation and fate of radiolabelled phosphate in Daphnia and the channel catfish.
5. Determine to what extent the phosphate responses seen in the catfish could be accounted for by the ionic nature of the phosphate ion complex. This will be accomplished by duplicating the cardiac, respiratory and biochemical experiments with the divalent sulfate ion.

CHAPTER II. MATERIALS AND METHODS

Chemical Monitoring

The first task to be undertaken in initiating this research was to ascertain the quality of the water in the Flat Creek embayment. This chemical monitoring was necessary to ascertain the pollution situation in the lake and to plan future experiments. In addition to measuring phosphate levels, several other selected parameters of water quality were monitored, according to procedures outlined in Standard Methods (31) and modified for the Hach DR-EL Field Kit.

Chloride is essential in the diet and passes through the digestive system unchanged to become one of the major components of raw sewage. Another source of chloride comes from chemicals added to detergents as water softeners. While high concentrations of chloride are not known to be toxic to man, efforts are made to keep chloride levels down in water supplies to avoid undesirable tastes. A mercuric nitrate method of analysis was used to quantitate chloride that involved a titration with diphenylcarbazone to yield a yellow to pinkish purple end point. Results of the test were expressed as milligrams of chloride per liter of water (mg/l).

Copper is not generally considered a health hazard, but in high concentrations it too can impart undesirable tastes to water supplies. Copper is found in natural waters, but usually industrial and sewage effluents are responsible for elevated levels. The Cuprethol Method for measuring copper which gives an intense yellow color in the presence of copper was chosen for use in this project. By spectrophotometrically examining the treated water sample, the copper concentration was quantitated as mg/l.

The color of water is dependent upon the nature of the suspended matter present. These suspended materials are usually metallic salts, organic matter and other dissolved matter which gives specific colors to the water depending upon pH. True color (as opposed to apparent color) is the color that remains after turbidity (see below) is removed by mechanical separation. Apparent color is determined with an untreated water sample. The quantification of color is based on the American Public Health Association (APHA) color unit defined as 1 mg/l platinum as chloroplatinate ion.

Turbidity is the result of suspended clay, silt, inorganic and organic matter, and micro-organisms such as plankton. A test for the turbidity of a water sample is a measurement of the optical properties of scattering and absorbing light by the particulate matter present. Quantification of turbidity is expressed as Formazin Turbidity Units (FTU).

Iron is not generally considered a health hazard, but a nuisance because it stains laundry and porcelain. Natural waters have varying amounts of iron resulting from the leaching of natural deposits. Iron from man-made sources can come from ironbearing industrial wastes or acidic mine drainage. Ferrous iron was determined by the 1,10-phenanthroline method. Results are expressed as mg/l ferrous iron (Fe^{+2}).

With silicon being such an abundant element, few water ways have absolutely no silica present. No toxic effect to humans occurs with high levels of silica. The Silicomolybdate Method involved the reaction of ammonium molybdate with silica and phosphate to form a yellow color. Oxalic acid was added to destroy the phosphomolybdic acid complex, but not the silico-molybdic acid complex. The remaining color was read directly and the results expressed as mg/l.

Sulfate is another chemical that appears in varying concentrations in natural waters. Industrial effluents frequently contain high levels of sulfates from the use of sulfuric acid. The presence of sulfate in water is necessary in the brewing industry to produce certain flavors. Determination of sulfate levels was done by a modification of the Barium Sulfate Turbidimetric Method. A silky white precipitant formed with the addition of the chemical and the amount of turbidity was proportional to the amount of sulfate present. Results were expressed as mg/l.

Measuring the pH of water to detect profound alterations from normal readings indicates the presence of strongly acidic or basic industrial wastes. Normal deviations of natural waters range from pH 4 to pH 9 and pH adjustment is frequently necessary in sewage treatment. The pH values were determined by using either a Corning Digital pH meter or colorimetric indicators whose colors were highly pH dependent.

Hydrogen sulfide results from the anaerobic decomposition of organic matter and is usually found in sewage and industrial waste waters. Hydrogen sulfide is as toxic as hydrogen cyanide, but its offensive odor is easily detectable long before toxic levels are reached. Hydrogen sulfide was determined by a method described in the Hach Chemical Company Manual for the DR-EL, p. 61, 1972. This test is not extremely sensitive, having as its lowest detectable point 0.1 mg/l. Results are expressed as mg/l hydrogen sulfide.

Nitrites occur as the intermediate step in the oxidation of biological compounds containing organic nitrogen. Bacteria convert ammonia under aerobic conditions to nitrites and in surface waters the nitrites are readily

oxidized to nitrates. The presence of high levels of nitrites indicates a source of heavy organic input since most nitrogen in water is in the nitrate form. Nitrites are used in industry as corrosion inhibiters and preservatives in food processing. Levels of nitrites were determined by using 1-naphylamine sulfanilic acid indicator. This test is very sensitive to nitrite concentrations and results are expressed as mg/l.

Nitrate is the most oxidized state of nitrogen found in water. Bacteria oxidize nitrites to nitrates under aerobic conditions. The most frequent sources of nitrate pollution come from incompletely treated biological wastes or runoff from heavily fertilized fields. Water quality is severely degraded by the high nitrate levels and the concomitant excess algal growth. The quantification procedure used in this project was the cadmium reduction method outlined in Standard Methods (31). Results are expressed as mg/l nitrate nitrogen. The results can be converted to mg/l nitrate (NO_3) by multiplying the mg/l nitrate nitrogen (N) by 4.4.

The monitoring of detergent levels in waterways gives a measure of the pollution from domestic sources. A common cause of frothing problems, surfactant detergents also endanger aquatic life. Anionic detergents were determined by the crystal violet method. Results are expressed as mg/l detergents LAS or ABS. LAS (biodegradable linear alkylate sulfonate) is used almost exclusively now, while ABS (non-biodegradable alkyl benzene sulfonate) has been largely eliminated from commercial detergents.

Phosphates are widely found in natural and waste waters. A certain amount of phosphate is necessary for the growth of many organisms in natural waters, but excessive amounts result in overfertilization. The major sources of phosphate come from domestic waste water, agricultural runoff, and industrial effluents.

Three forms of phosphate are widely distributed in water; orthophosphate, meta-(or poly-) phosphate, and organically bound phosphate. Phosphate concentrations were determined by using a modification of the molybdenum blue procedure. Since meta-phosphate will not register in this test, one test is conducted for ortho-phosphate and another is conducted for total inorganic phosphate. By subtracting ortho from total phosphate, meta-phosphate levels may be determined.

One of the most important tests in determining the quality of water is the measurement of dissolved oxygen (DO). The DO of a body of water is indicative of the amount of waste oxidation taking place. High DO levels indicate clean water with self purification capabilities intact. Low DO readings show that organic loading is taking place and oxidation of this waste is incomplete. Maintaining at least a minimal DO level at sewage treatment plants results in optimal oxidation in the shortest possible time. For the determination of dissolved oxygen, the Alsterberg (azide) modification of the standard Winkler method was used. Results were expressed as mg/l dissolved oxygen.

Bacteriological monitoring was conducted as prescribed by the American Public Health Association's Standard Methods for the Examination of Water and Waste Water (31). This method defines the test for the coliform bacteria that are found in domestic sewage. A lactose broth was used as a presumptive test in which a water sample was placed in contact with the medium and the presence of gas evolution monitored. Confirmative tests were conducted using Brilliant Green Lactose Bile Broth. Transport of the bacteriological samples was accomplished within one hour and conformed to the methods set forth in Standard Methods.

One liter bottles were used in collecting chemical samples after having been washed thoroughly and rinsed several times with distilled water. Mercuric chloride and sodium chloride were added after the distilled water rinse to destroy any microorganisms that would alter the composition of the chemicals in the sample being quantitated. Erroneous, elevated DO values result from tests done on transported water samples, therefore, this test was done at the test site at the same time the water sample was taken. The water samples were rapidly transported to the lab and stored at $5^{o}C$ until the remainder of the chemical tests could be performed. All measurements of these water quality parameters were conducted using the Hach Field Engineer Kit. Samples were taken from the same test site over the 3 year-course of the investigation (see Figure 1). Water samples were taken monthly, when possible, during the course of the three year project. To determine if significant changes in the water quality parameters occurred over the total five years that there is available data, the Wilcoxon Rank Sum Test was employed.

Upon taking the sample, the date was recorded as well as the water and air temperature. Also noted on the individual data sheet for each sample was the date the remaining chemical tests were performed. Identical sampling methods were used in the two 48 hour monitoring periods except the samples were taken hourly.

Fish Storage Tanks

As the water sampling was begun, initial steps were taken on the procurement and establishment of the facilities to maintain the experimental

organisms. Figure 2 shows the system in which the channel catfish were kept. The 750 gallon fiberglass storage tanks were acquired from Spectrum Plastics of Lebanon, Tennessee and placed in heated rooms near the School of Biology at the Georgia Institute of Technology. A Calgon Model 12AB activated charcoal filter was purchased and used to condition the water that entered the tanks. All plumbing used to install the system was made of polyvinyl-chloride (PVC) and joints were sealed with PVC cement. Tap water was run through one inch PVC pipes to the filter and then into a reservoir tank that was positioned above three other 750 gallon tanks where the fish were kept. This tank was used to keep the other three full and allowed the water to "age." This process removed what chlorine the filter did not.

The three fish tanks had two inch diameter PVC pipes approximately 30 inches long fitted to center drains. This allowed a continuous flow of water to enter the tank and kept the depth at 30 inches. Total overturn of the water was estimated to take 8 hours with water temperatures averaging 15 to 20^{o}C for the three year period fish were maintained.

Experimental Organisms

Channel catfish, Ictalurus punctatus, were obtained from a variety of sources. Two different sizes were employed in the experimentation. Large fish, approximately 15 to 30 cm, were used in enzyme, labelling, cardiovascular, metabolic, and food conversion studies. Small catfish (fingerlings) approximately 4 to 6 cm were also used in labelling experiments. Adult fish were obtained from: Cohutta National Fish Hatchery, Cohutta, Georgia; King Mountain Hatchery, Thomaston, Georgia; Auburn University Aquaculture Department, Auburn, Alabama; Hershel Webster Fish Farm, Cleveland, Georgia, and

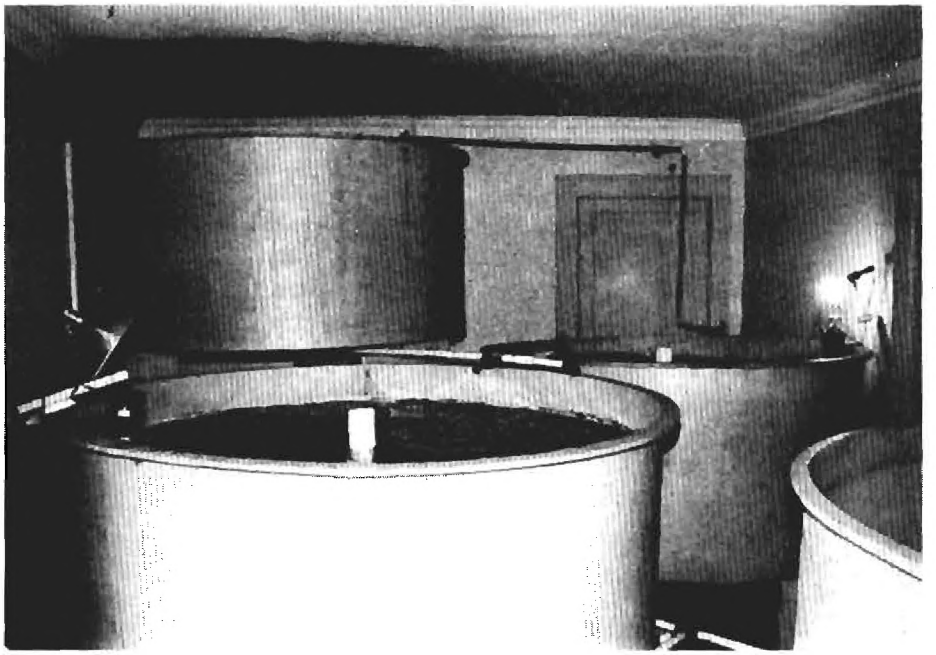


Figure 2: Tank system in which catfish were maintained

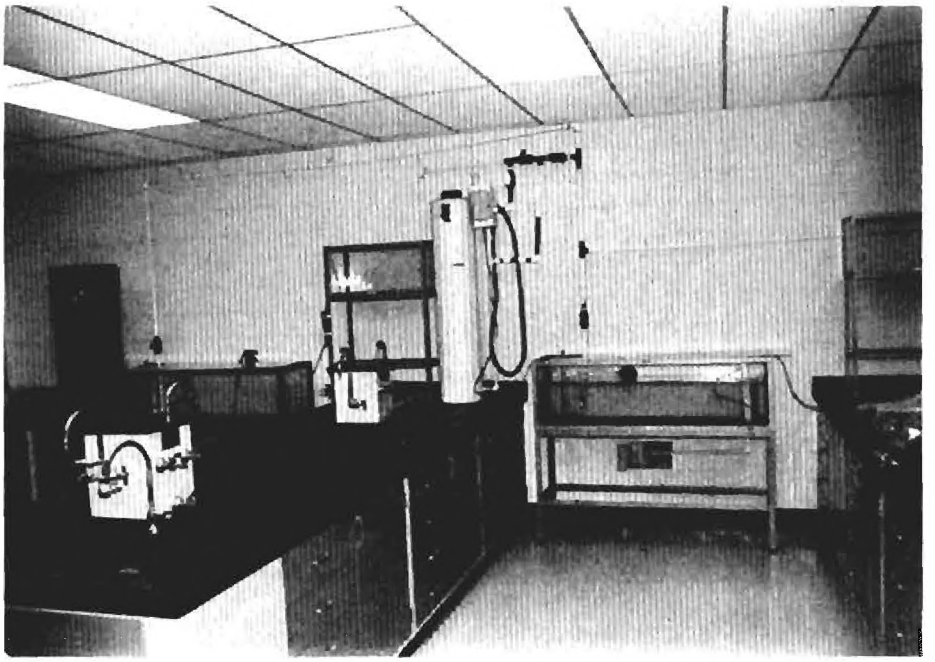


Figure 3: Experimental tanks used to expose catfish to phosphate

Jasper National Fish Hatchery, Jasper, Alabama. The fingerlings were acquired from the Cohutta National Fish Hatchery. Transportation of the fish was accomplished as rapidly as possible by lining 30 gallon garbage cans with plastic bags and bubbling either oxygen or compressed air slowly through the water. Fish were fed ad libitum once every two days with Purina Trout Chow and received a twelve hour light, twelve hour dark light regime. The water was constantly aerated with a Mino-Saver water aerator. Daphnia were acquired from Carolina Biological Supply of Burlington, North Carolina as needed. These zooplankton were taken from their shipment vials and placed directly into the experimental tanks.

Experimental Tanks

The experimentation in this project was accomplished with the use of four 21-gallon and four 75-gallon Jewel aquaria (Chicago, Illinois). These tanks were purchased from Carolina Biological Supply Company of Burlington, North Carolina, along with tanks stands and air pumps. To study the effect of phosphate stress on the fish, a tank system as shown in Figure 3 was used. Three small tanks were placed approximately one meter above three of the large tanks. A stock solution of phosphate with a concentration of 10 g/l was kept in these smaller aquaria. Siphons tubes were placed in the small tanks and connected to Lab-Crest Mark III flowmeters (Fisher Scientific) (Figure 4). Other tubes were run from the flowmeters to the edge of the large tanks and positioned so the stock phosphate solution could drip into the larger tanks. A second Calgon model 12AB activated charcoal filter fitted with PVC plumbing

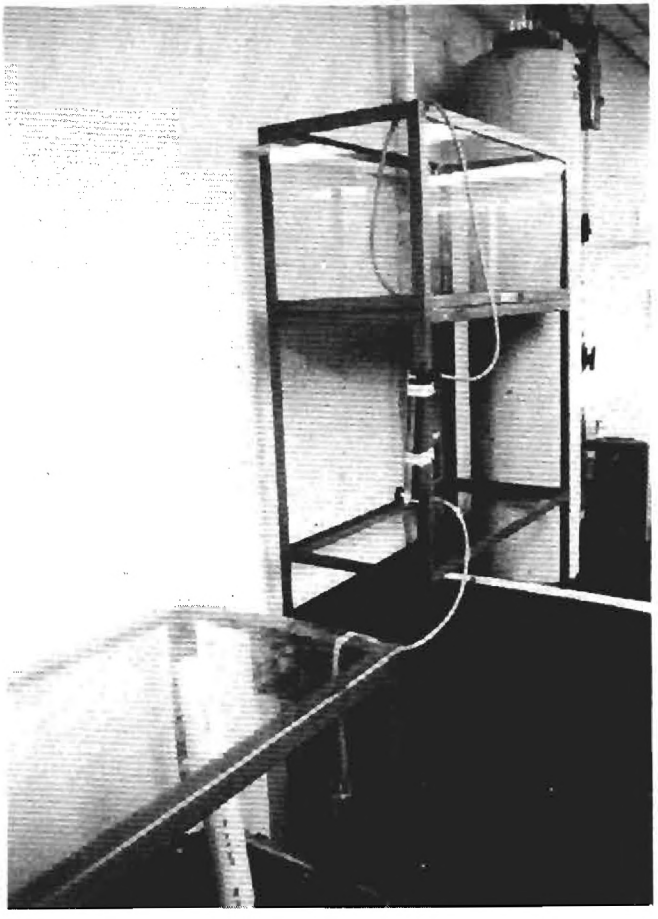


Figure 4: Phosphate exposure system - stock solution tank, flowmeter and experimental exposure tank.

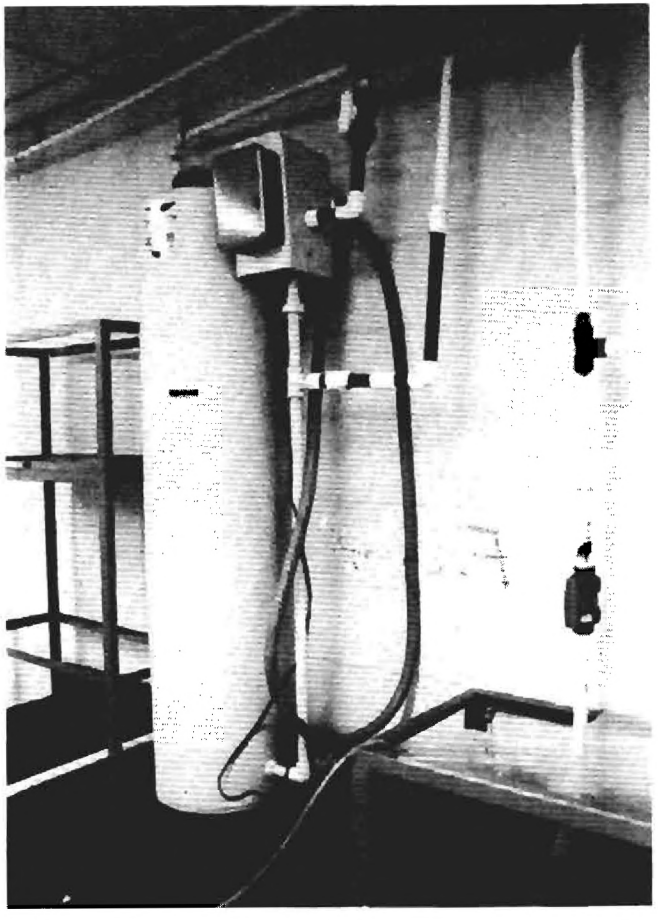


Figure 5: Activated charcoal filter used to condition water

was used to condition the tap water supplied to these tanks (Figure 5). PVC toilet tank cut-off valves were adjusted to supply a one-liter-per-minute flow of the fresh water to the tanks. Pipes, 26 cm long, were fitted into the drains of the large tanks, maintaining the water level at this depth. Turnover time for the 75 gallon tanks was just under five hours. Considering the flow rate of the freshwater through the 75 gallon tanks and the phosphate stock solution concentration in the smaller elevated tanks, the flowmeters were calibrated to maintain the proper phosphate concentration while allowing a continuous turnover of the water. Fish exposed to phosphate in this manner were used for cardiovascular, food conversion efficiency, and enzyme studies. The same feeding, light, and aeration practices employed for the fish storage tanks were used in the experimental tanks in all but the food conversion studies. Exposure of fish to sulfate was done with a concentration of sulfate over a short period of time that would represent an acute insult, hence the continuous flow system was not employed. The four 21 gallon tanks were used in this experiment, but the water was not changed. Details of both the phosphate and sulfate experiments are discussed below.

Due to the need of avoiding radioactive contamination, radiotracer studies using sulfur-35 and phosphorus-32 were conducted in a single 21 gallon tank. The fish were not involved in the radiotracer studies long enough to require feeding, but continuous aeration was necessary. After the tracer studies were completed the tank was scrubbed with Industrial Brand Antiradioactivity Cleaning Compound (Industrial Colloids and Chemicals, Incorporated, Knoxville, Tennessee) and put in a safe place until radioactivity was not detectable.

Biological Monitoring

Effect of Phosphate on Cardiac and Opercular Rates

To assess the effect of phosphate on the cardiac and opercular function of catfish, several replications of the same basic experimental format were carried out. This format was developed by trial and error experimentation, resulting in an experimental design that would, (1) provide an easily manipulatable system allowing chronic phosphate exposure of catfish, and (2) allow for a continuous flow water system to be developed. Various methods were attempted to tag the fish, but any device implanted on the fish's body resulted in a necrosing of the underlying tissue and a loss of the tag. Fin clipping was not satisfactory because it did not permit the identification of a large enough number of fish. Finally, two millimeter long pieces of colored plastic wire insulation were used in different permutations on the pectoral girdle spines or dorsal spine for identification.

Fish were placed in the tank system described previously and allowed to acclimate in fresh filtered water for one to two days. Prior to exposure to phosphate, two fish were sacrificed for control cardiac and opercular rates. The flowmeters were then calibrated to maintain a phosphate level of 5 mg/l in the 75 gallon tanks. This concentration was maintained for 24 hours. At the end of this 24 hour period, two more fish were sacrificed for enzyme studies and the same two fish were monitored for changes in heart beat or opercular rhythms. The flowmeters were then recalibrated to deliver enough phosphate stock solution to maintain a concentration of 10 mg/l. 24 hours later, two

more fish were sacrificed and the two fish monitored previously were again examined for cardiac or respiratory changes. This process was repeated with phosphate concentrations of 15 and 20 mg/l in similar 24 hour intervals. After removing the fish at the 20 mg/l concentration, the concentration was not further increased. Fish were allowed to remain in the 20 mg/l concentration and were removed at 72 hours and 2 weeks from the time this concentration was achieved. A step by step diminution of the phosphate concentration was then conducted in a similar fashion. After the phosphate was cleared from the tank, two final fish were sacrificed for enzyme studies and the two fish studied throughout the experiment were monitored one last time. This method gave insight into responses of the catfish to chronic phosphate exposure.

To investigate the effect of acute phosphate exposure on the cardiac and opercular rates of the catfish, two separate exposure regimes were carried out using this tank system. Eight fish were placed in a tank and allowed to acclimate for several days. After control readings were taken with no phosphate in the water, enough phosphate stock solution was added by the flowmeter to bring the water in the tank up to a concentration of 2 mg/l. The fish were monitored after they had been at this concentration for one hour. The flowmeters were then recalibrated to bring the phosphate concentration up to 4 mg/l where the fish remained for another hour. Eight fish were monitored at this concentration and this procedure was continued in 2 mg/l increments until a final concentration of 20 mg/l was reached. At a later date, another acute exposure regime was carried out using identical procedures except the phosphate concentrations were increased in 20 mg/l increments until a final concentration of 160 mg/l was attained. Exposure times were again one hour.

To record the cardiac and opercular rhythms of these catfish, a chamber was constructed of 12 mm thick Plexiglas having the dimensions of 32 cm in length, 8.5 cm in width, and 19 cm in height (Figure 6). All exterior surfaces were painted black to reduce external stimuli. This chamber gently restrained the fish while allowing it to swim freely, but at the same time maintained the long axis of its body in one direction. A black Plexiglas lid was placed over the chamber to shield the fish from movements of the operator. A continuous flow of water was maintained from a 75 gallon aquarium via a tube entering the chamber near the caudal fin of the fish. This water was returned to the 75 gallon tank by a drain at the opposite end of the tank that kept a water depth of approximately 8 cm deep. This provided fresh, oxygenated water throughout the experimental period.

Carbon electrodes, 25 mm in diameter and 3 mm in thickness were permanently attached to each side of the tank at the level of the opercula. Leads from the electrodes were attached to either a Narco Bio-system DNP-4B Physiograph or a Grass Model 7B Physiograph (Figure 6). Electronic filtering eliminated undesirable swimming movements, high frequency electrical noise and 60 cycle noise. The Narco Bio-System was utilized for most of this work. The transducers were returned to the manufacturer in Houston, Texas for electronic filtering modification as outlined by this investigator. For the channel recording opercular movement, the high filter was set at 3 or 10 hz and the low filter at 1 or 3 hz. Amplitude of the signals was controlled by a variable switch set at 2.5 or 10 mV/cm, depending on the size of the fish. Most of the large fish required an amplitude setting of 5 or 10 mV/cm, while the small fish required a setting of 2 mV/ cm. Water resistivity was approximately 4,600 ohms

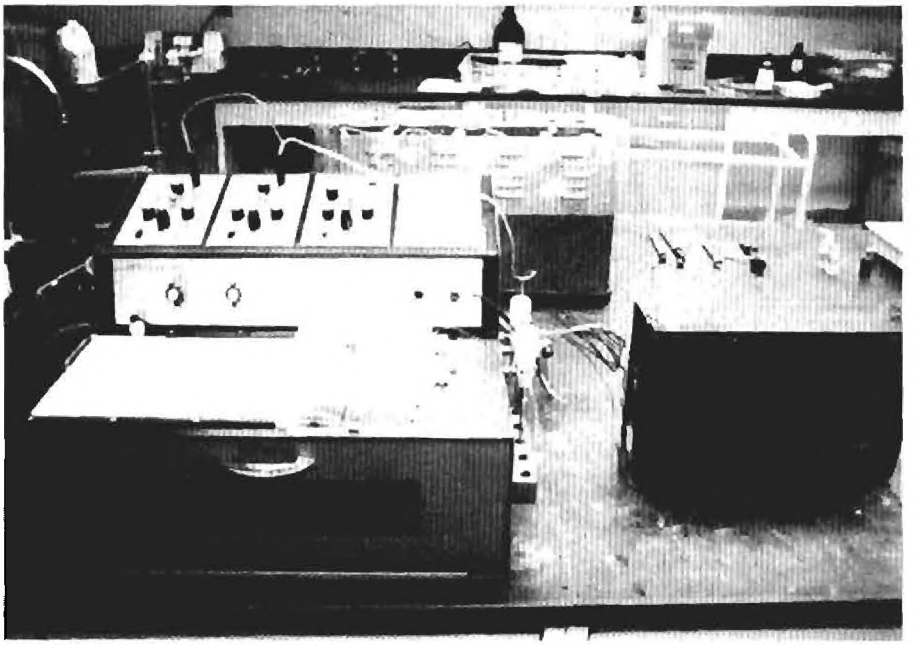


Figure 6: Physiograph and chamber used to monitor cardiac and opercular rates

Figure 7: Apparatus used to monitor oxygen consumption.

(0.22 millimho/cm). Cardiac beats were recorded on one channel of the physiograph and opercular beats on another. A timer on the physiograph marked one second intervals. Catfish weights ranged from 170 gm to 450 gm.

Effects of Phosphate on Respiration

Since oxygen consumption is an indirect but accurate measure of metabolic rate (32), changes in the consumption rate of O_2 are indicative of metabolic aberrations. Insight into the effect of different concentrations of phosphate on catfish metabolism was gained by such a method. A second chamber was constructed from Plexiglas that was 25 cm long, 8.5 cm wide, and 19 cm high. The top of the chamber was fitted with a cork gasket upon which a 12 mm thick lid could be fastened to give a watertight seal (Figure 7). All exterior surfaces were again painted black and a 33 mm diameter hole was bored in the center of the lid. A Clarke type oxygen electrode was fitted in the center of a size 8 rubber stopper which was in turn fitted into the hole atop the lid, thus maintaining a watertight seal. The Chamber could be sealed without air being trapped inside by submersing it in an 11 gal aquarium filled with water to a level 10-15 mm above the lid of the chamber. This allowed the lid to be placed on one edge of the chamber gasket and lowered gently to fit tightly on the entire gasket without trapping air inside. This method also kept the non-immersible portion of the electrode from getting wet. A plastic petri dish was trimmed and glued to the bottom of the chamber and a magnetic stirring bar was placed inside of it. When a magnetic stirrer was placed below the aquarium, a continual mixing of the water in the chamber could take place without the fish touching the stirring bar. The electrode was connected to a Keithley Instruments Model 610C Electrometer which was connected to a Grass Model 7DAE

Physiograph to record the oxygen consumption. A YSI Telethermometer (Yellow Springs, Ohio) was also connected to the Physiograph to give a record of the temperature in the aquarium since metabolism is affected by temperature changes. Because of this, the temperature of the water in which the fish were kept, and the temperature of the water in the chamber was kept at $25 \pm 0.5^{o}C$ in a carefully regulated room.

Three fish, ranging in weight from 105 to 174 g , were used in this phase of the project. Each fish was placed in the chamber with fresh, aerated, filtered water and the stirrer was started. Control O_2 consumption rates were taken for a period of at least 15 minutes for each fish. Knowing the volume of the water in the 75 gallon aquaria, enough monobasic sodium phosphate was weighed out to make a concentration of 5 mg of phosphate per liter of water. After the control O_2 consumption rates were taken on the three fish, they were placed in this tank for a period of 24 hours. There was no water flow during this period, but continuous aeration was maintained. After 24 hours at 5 mg/1, O_2 consumption rates were again taken on each of the three fish. Without a turnover of water in the tanks, fish waste products would build to toxic levels if too long a period of time passed. For this reason, after O_2 consumption rates were determined, the fish were placed in another 75 gallon tank. This second tank has continuously aerated fresh filtered water to which enough sodium phosphate had been added to achieve a concentration of 10 mg/1. O_2 consumption rates were determined as before and concentrations of 15 and 20 mg of phosphate per liter of water were also used.

Food Conversion Efficiency

The effect of phosphate on food conversion was examined on a total of 18 fish in two treatment groups. Nine other fish served as controls. Three 75 gallon tanks were used in this study and all of them received a continuous supply of fresh filtered water. Aeration was maintained throughout the course of the experiment. The first tank served as a control tank, therefore, it received no phosphate. The second and third tanks were the treatment tanks, therefore, a phosphate drip was initiated in each one to maintain a phosphate concentration of 10 and 20 mg/l respectively. The lengths of all fish were recorded; they were weighed, tagged, and placed in the proper tanks with a 12 hour light, 12 hour dark photoperiod regime. The fish were fed Purina Trout Chow ad libitum and a record of the quantity of food consumed was kept for a period of 6 weeks (42 days). At the end of this period of time, the fish were weighed and measured and the percentage change was computed. Analysis of variance was employed to evaluate the results.

Biochemical Monitoring

To find what effect phosphate had on metabolism, two different characteristics of metabolic enzymes were examined; the basic organization of enzyme molecules and the nature of the electrical charges found on the molecules.

All enzymes are made of protein and proteins are made of basic building blocks called amino acids. Amino acids are linked together by peptide bonds to form polypeptide chains. The sequencing of these amino acids determines the primary structure of the enzyme. The secondary structure of these protein chains is the coiling or sheet like structures formed by the stacking and

interaction of the polar and charged side chains found on the amino acids. The folding of the coils of protein back upon itself is the tertiary structure. This structure is also maintained by interaction of amino acid side chains. Activity of an enzyme is determined more by tertiary structure than anything else, but many enzymes do not achieve activity until they have all subunits assembled together. This association of subunits is the quaternary structure of proteins which gives optimal enzyme activity.

In an enzyme system that exhibits a subunit type assembly, stress placed on the system may be manifest by changes in the ways the subunits associate.

LDH Electrophoresis

In mammals, the enzyme LDH has a structure which consists of 4 subunits of two types. Heart (H) and muscle (M) subunits may combine to give H_4, H_3M, H_2M_2, H_1M_3, and M_4 type combinations. Not all organisms possess exactly 5 combinations. Some possess many more and some have less. The only LDH isozyme system which is adequately described in fish, in terms of subunit structure, is that of the salmonids (33,34). In fishes, the number of LDH isozymes have been reported to vary from one to about 20 (33,35).

Characteristic concentrations of each of these combinations are found in normal organisms and by the use of electrophoresis, changes in the patterns from a normal state can be detected. Electrophoresis takes advantage of the fact that these protein subunits have characteristic changes on them by nature of the amino acid side chains. The number of charges on an enzyme molecule depends upon the subunits that make it up and the pH of the environment.

In the use of electrophoretic technique, a substrate is supplied upon which a small sample containing the enzyme can be placed. A current is then passed through the substrate and the enzymes are separated based on their

different mobilities in an electric field.

After catfish had been exposed to phosphate as described previously, they were sacrificed and the hearts were removed. The net weight of the organ was recorded and it was quickly frozen using dry ice and acetone and stored at -20°C for later enzyme analysis.

A crude extract containing the LDH enzyme was prepared by placing each sample in 1 ml of distilled water and thoroughly homogenizing it in a glass homogenizor with a motor driven teflon pestle. All samples were kept cold in an ice bath to avoid excessive enzyme denaturization. The homogenate was then placed in a refrigerated centrifuge (Sorvall Model RC-2) and centrifuged at 2000 x g for 45 minutes. The supernatant was decanted and used for the enzyme assay.

Two electrophoretic systems were used to study the LDH enzyme profiles. Cellulose acetate strips were used initially with a barbitol buffer system. Further work was done using hydrolyzed potato starch gels with three different buffer systems.

Sepraphore III brand cellulose acetate strips (Gelman Corporation, Ann Arbor, Michigan), measuring 2.5 x 15.2 cm, were used in a Gelman electrophoresis buffer tray with a pH 8.6 barbitol buffer of 0.075 ionic strength. After the strips were allowed to soak in the buffer for several hours, six to nine microliters of the extract were applied in a thin line with a Gelman serum applicator. A potential of 300 volts at 3-4 milliamps per strip was supplied by a Heathkit Model IP-17 power source for a period of 60 minutes.

Visualization of enzyme activity in the strips was achieved by placing another cellulose acetate strip that had been soaked in a solution made of

the reagents in Appendix I on top of the electrophoresis strip. The two strips were then sandwiched between two glass microscope slides and incubated in the dark at 37°C for one hour. The staining strip usually best revealed the banding patterns.

Quantification of the enzyme bands present on the strip was done spectrophotometrically. To do this, both the electrophoretic and the staining strip for each sample were cleared by soaking them in Gelman SepraClear II (40.0% aqueous N-methyl pyrrolidone V/V) for five minutes. The strips were again placed on microscope slides, the excess clearing fluid was removed, and they were dried for 20 minutes at 80-100°C. The strip was removed and allowed to cool undisturbed. Each band was cut from the strip after being peeled from the glass and dissolved in three ml of a 50% glacial acetic acid and 50 % acetone mixture. This solution was placed in a cuvette and the optical density determined using a Spectronic 20 (Baush and Lomb) at 570 nanometers (nm). By this method, the percent of the total LDH activity for each band could be determined.

The same LDH extraction methods were used in the starch gel electrophoretic studies, but three different buffer systems, each of a different pH, were tried in an attempt to improve resolution of the bands.

Two gel trays 15 x 20 cm in size, were constructed of 5 mm thick Plexiglas sheets. The trays were 4 mm deep and the starch gels that were made in them were not removed until just prior to staining.

Hydrolyzed potato starch was obtained from Sigma Chemical Company (St. Louis, Missouri) and was used initially to make a 10% gel. Gels made of this concentration were found to lack structural integrity and were difficult to handle without destroying. A 20% gel was found to withstand the manipulations

required by the staining procedure and gels were made in this concentration from all three of the buffer systems. Different dilutions of each of the buffers were used in making the gels. These dilutions along with cathode and anode dilations appear in Appendix II.

The three buffer systems used were: edtate-borate-tris, pH 8.6; tris-citrate, pH 6.9; and tris-verseve-borate, pH 8.0.

Gels were made by adding 32 g of starch to 160 ml of buffer in a 2 liter flask. This solution was heated until it gelled at which time a vacuum was rapidly applied to it to remove all gas produced. The heated gel was poured into the gel tray and before it solidified, a dissection needle was used to remove any large bubbles that remained after aspiration. After the gel cooled, a cut was made 4.5 cm from the cathodal end. Into this slit were placed 1 x 3 mm pieces of filter paper that had been soaked in the LDH extract. The gel trays were placed in a Gelman electrophoresis buffer tray and a piece of 5 x 14 cm filter paper was placed on each end of the gel so that 2 cm of the paper contacted the gel and the other end rested in the buffer. The entire tray was then placed in a refrigerator at $5^{o}C$ and a potential of 300 volts with 10 milliamps current was applied to the gel for 12 hours. After the current was turned off, the gel was sliced lengthwise into two 3 mm-thick gels of the same dimensions and placed in a staining dish with the stain preparation in Appendix I. Incubation of the gels was carried out in darkness at $37^{o}C$ for 3-4 hours.

Enzyme Activity

The optical property of absorbance was used to measure the effect of phosphate on enzyme activity in the catfish. The cofactors $NADH^{+} + H^{+}$ and $NADPH^{+} + H^{+}$ both absorb light strongly at 340 nm. The oxidized forms of these

two cofactors do not absorb light as readily. LDH requires $NADH^+ + H^+$ (reduced form) as a cofactor in catalyzing the conversion of pyruvate to lactate. Because of this, enzyme activity is proportional to the change in absorbance as $NADH^+ + H^+$ is oxidized to NAD^+. While neither PyK or HK require either of these two cofactors for catalysis, they are still used to measure enzyme activity. The products formed by both reactions are immediately acted upon by another enzyme in the reaction solution that does require one of these cofactors. This is an indirect, but accurate method of measuring PyK and HK activity.

In the assays of these three enzymes, the rate of the reaction (change in absorbance) is directly proportional to the amount of enzyme present.

LDH assay

The method of phosphate exposure to fish as well as the method of extracting LDH has been discussed previously. Extracts from each tissue sample were made and kept on ice to minimize denaturation. The activity of LDH, or the number of units of enzyme per ml of solution, was calculated using the equation in Appendix III. This value was normalized by the volume of the extract and the weight of the heart tissue in order to calculate the units of LDH per mg of heart tissue. A Beckman Model 25 digital spectrophotometer with recorder was set at 340 nm and used in all enzyme activity assays. The reagents in Appendix IV were added to both the reference cell and sample cell of the spectrophotometer and the instrument was zeroed. A 0.01-ml volume of the enzyme extract was added to the sample cell and the absorbance was monitored for a period of time necessary for a significant change to take place. Addition of the enzyme extract to the sample cell gave a total volume of 2.98 ml which means the dilution factor used in the equation in Appendix III would be one two hundred and ninety eighth (1/298). Student's t-test was used to analyze

the effect of treatment.

To study the effects of sulfate on LDH activity, the same procedure as described above was used. The only difference was that sulfate exposure was administered to the fish on an acute basis instead of phosphate.

PyK and HK assay

The catfish liver was selected to determine what effect phosphate and sulfate had upon PyK and HK activity. A crude extract containing both PyK and HK activity was prepared by weighing the liver tissue and homogenizing it in 1 ml of 0.5% EDTA. Ice baths were used to reduce the loss of enzyme activity. After homogenization, 0.26 g of ammomium sulfate was added to each ml of the supernatant to attain an ammonia concentration of 1.75 M. After being allowed to sit for three hours, the solution was centrifuged at 2200 x g for one hour. The supernatant was then decanted and allowed to sit for two hours after adding 0.105 g more of ammonium sulfate per ml of solution. After another hour of centrifugation at 2200 x g, another 0.037 g of ammonia sulfate per ml of solution was added. One last centrifugation at 2200 x g for one hour completed the extraction procedure and the final volume of the supernatant was recorded.

The reaction catalyzed by PyK begins with phosphoenolpyruvate (PEP) and converts it to lactate with a concomitant formation of ATP from ADP. Excess LDH in the reaction mixture then converts lactate to pyruvate along with the oxidation of $NADH^+ + H^+$ to NAD^+ which can be followed spectrophotometrically.

The Beckman model 25 was zeroed after the reagents in Appendix V were added to both the reference and sample cells. A 0.1 ml volume of the enzyme extract was then placed in the sample cell and absorbance readings were taken every 30 seconds for approximately 8 minutes. The equation in Appendix III

was used to calculate the activity of the enzyme using a dilution factor of one to three hundred and one (1/301). The enzyme solution volume and liver tissue weights were used to calculate the enzyme activity present per mg of tissue.

The reaction catalyzed by HK involves the phosphorylation of glucose at the number six carbon and results in the production of glucose-6-phosphate. To quantitate the activity of this enzyme, excess glucose-6-phosphate dehydrogenase and $NADP^+$ are added to the reaction mixture. Glucose-6-phosphate dehydrogenase removes two hydrogen atoms from glucose-6-phosphate and transfers them to $NADP^+$ to form $NAHPH^+ + H^+$. The reduced form of this dinucleotide absorbs light strongly at 340 nm, so as the total reaction in the cuvette proceeds, there is an increase in absorbance.

Using the reagents in Appendix IV in both the reference and sample cells, the Beckman Spectrophotometer was again zeroed. Into the sample cell was added 0.1 ml of the enzyme extract and the change in absorbance was monitored at 30 second intervals for approximately 8 minutes. The enzyme activity was calculated by using the equation in Appendix III and a dilution factor of one three hundredth (1/300). The enzyme activity, enzyme extract volume and liver tissue weight were used to calculate the quantity of enzyme present per mg of tissue weight.

Radiolabelling Studies

Radiolabelling studies designed to examine the uptake and storage of phosphorus in the Flat Creek ecosystem were done with three different experiments.

The uptake of phosphorus in *Daphnia* alone was examined first. *Daphnia* acquired from Carolina Biological Supply were in small plastic vials that, according to the supplier, contained enough *Daphnia* for a class of 25. The

actual number was approximately 50 to 60 organisms. The experimentation took place in the vials after the water in which the Daphnia arrived was decanted to 49.5 ml. Three vials received 0.5 ml of distilled water to make a total of 50.0 ml, and from these vials 1.0 ml of the resulting mixture was added to 9 ml of Aquasol cocktail (New England Nuclear). These 3 vials served as a background reference to which the phosphorus uptake in the Daphnia was measured. Ten millicuries (mCi) of $H_3P^{32}O_4$ in 1 ml of H_2O were acquired from New England Nuclear and diluted to 20 ml with distilled water. A 0.5 ml (0.25 mCi) of the P-32 was added to each plastic vial to bring the total volume to 50 ml. A total of 36 vials received the radiolabelled phosphorus, three of which had 1 ml of the water alone added to Aquasol to serve as standards. After introducing the P-32, Daphnia were removed from 3 vials at the following in time periods: 3, 5, 10, 15, 30 minutes, and 2, 4, 8, 24, 48 hours. The Daphnia were removed by filtering the contents of each vial with a Millipore Filter (Millipore Corporation, Bedford, Massachusetts). The Daphnia were then dried, ground up, dissolved in 1 ml of distilled water and added to 9 ml of Aquasol. All samples were counted on a Beckman Model LS 100C liquid scintillation counter 3 times for 3 minutes.

Catfish uptake of P-32 was the next subject investigated. A 21 gallon aquarium was set in a lighted, ventilated, safety hood and filled with 20 liters of water. The water was continually aerated and 8 catfish of approximately 170 to 190 g in weight were allowed to acclumate for two days. Two fish were sacrificed prior to introducing the P-32 and the following organs were removed: bone (from pectoral fin), brain, gill, heart, kidney, liver, muscle (section of the body wall), and stomach. Subsequently, 10 mCi of P-32 (New England Nuclear) were added to the water to give a concentration of 1.11×10^6

disintegrations per minute per ml (dpm/ml). Two more fish were sacrificed at 1 day, 1 week, and 2 weeks and the same organs were removed. All organs were homogenized in an equal-weight volume of Frog Ringers and diluted 10 times with the Ringers. From each tissue homogenate, 0.2 ml was added to 9.8 ml of Aquasol and mixed. Each sample was placed in a Beckman liquid scintillation counter and counted 3 times for 3 minutes.

The third experiment was designed to examine the fate of phosphorus in the Flat Creek ecosystem and consisted of feeding Daphnia that had been allowed to incorporate P-32 to catfish that had had no previous P-32 exposure.

Into a 21 gallon tank, 20 liters of fresh aerated water were placed with 40 small catfish ranging in weight from 40 to 70 g. Due to the precautions and complexities involved in this experiment, this was 5 fish more than necessary for the experiment in case any fish died. At the same time, another 21 gallon tank was filled with 10 liters of water to which was added 60 vials of Daphnia acquired from Carolina Biological Supply. This represented approximately 3000 to 3500 Daphnia. To the water in this tank, 20 mCi of P-32 was added and the microorganisms were allowed 2 days to achieve a balance between the P-32 present in the water and the P-32 taken into their bodies. It was assumed that a balanced state would be achieved in this period of time.

To initiate the study, 5 fish were taken from the fish tank and the following organs were removed: bone(from pectoral fin), brain, gill, heart, kidney, liver, muscle (section of body wall), and stomach. After the organs were homogenized in an equal-weight volume of Frog's Ringers, 0.2 ml of the homogenate was added to 9.8 ml of Aquasol. In this experiment, the homogenate was not diluted 10 times as before because of the very low levels of exposure

to which the fish were subjected. These 8 organs from 5 fish gave a measure of the background irradiation found during this experiment. After the control fish were sacrificed, 1.67 l of water from the Daphnia tank were filtered with a Millipore filter and the microorganisms were placed in the tank with the fish. It was assumed that this volume of the Daphnia tank would contain from 500 to 600 Daphnia. After filtration, the water was returned to the Daphnia tank. Three days after the Daphnia were placed in the fish tank, 5 more fish were sacrificed. After removing the organs from these fish, 2 l of water from the Daphnia tank were filtered and the microorganisms were placed in the fish tank. Fish were subsequently sacrificed at day 5, 8, 10, 12, and 14. From 500 to 600 Daphnia were placed in the fish tank on day 5, 8, 10, and 12 by filtering 2.5, 3, 5, and 10 l of water from the Daphnia tank respectively.

All samples were counted on a Beckman liquid scintillation counter 3 times each for 2 minutes.

CHAPTER 3. RESULTS AND DISCUSSION

Chemical Monitoring

The data resulting from the investigations of the water quality at the Flat Creek embayment are presented in Table I and Appendices VII and VIII. Table I lists the ranges of the yearly values for the water quality parameters examined and the means of these values were compared by the Wilcoxon Rank Sum Test. The first two columns of data in Table I have been taken from OWRR Project Number A-035-GA which was completed in January of 1974 by the investigator of the current project. These columns represent the years 1971 and 1972. The last three columns contain the same data for the years 1973, 1974, and 1975, plus chloride and copper tests, which were not performed in 1971 and 1972.

Appendix VII lists the values for the water analyses conducted during the first 48-hour monitoring period which took place in July of 1974. Appendix VIII contains the water quality parameter values obtained from the second 48 hour monitoring period which was conducted in May of 1976.

In Table I, for the five year period that data was available, pH, nitrite, and temperature values are not significantly different from one year to the next. Overall water quality appears to be best in 1972. The only values in 1971 that were statistically lower than 1972 were nitrates and undissolved oxygen, the remaining values being higher. In 1973, nitrate, ortho-phosphate and dissolved oxygen in addition to the pH, nitrite and temperature values were not significantly different from 1972. All other values in 1972 were lower than 1973 and none were higher. Silica and meta-phosphate are the only two values in 1973 that were higher than 1974.

Table 1

WATER ANALYSIS OF FLAT CREEK EMBAYMENT

	1971[a]	1972[a]	1973[a]	1974[a]	1975[a]
Color APHA Units	29.00-230.00[c]	0.00-45.00[d]	85.00-129.00[d]	140.00-185.00[b]	60.00-230.00
Turbidity FTU	27.00-60.00[c]	1.00-28.00[d]	25.00-48.50[d]	40.00-91.00[b]	4.90-140.00
pH	7.20-8.60[b]	6.95-8.54[b]	5.00-8.60[b]	6.40-7.50[b]	6.40-6.70
Detergents mg/l	0.20-0.40	0.00	0.00-0.40	0.00	0.00
Iron mg/l	0.89--1.55[c]	0.40-0.60[d]	1.10-1.30[b]	0.37-1.68[b]	0.89-2.01
Nitrate mg/l	1.10-2.20[d]	2.00-3.00[b]	0.20-8.50[d]	8.30-12.00[c]	0.00-4.20
Nitrite mg/l	0.19-0.32[b]	0.10-0.45[b]	0.07-0.27[b]	0.00-0.15[b]	0.06-0.34
Orthophosphate mg/l	10.00-13.00[c]	0.10-8.00[b]	5.00-17.00[b]	7.00-12.00[b]	2.50-11.80
Metaphosphate mg/l	1.00-4.00[c]	0.00-0.80[d]	2.00-4.00[c]	0.50-2.25[b]	0.50-10.0
Total phosphate mg/l	13.00-50.00[c]	0.10-8.50[d]	9.00-28.50[b]	9.00-36.00[c]	4.25-13.50
Silica mg/l	10.00-17.00[c]	2.40-3.40[d]	9.40-20.00[c]	5.50-6.50[d]	13.10-150.00
Dissolved oxygen mg/l	4.85-7.25[d]	8.00-11.00[b]	5.50-11.70[b]	2.00-10.50[b]	1.00-12.00
Chloride mg/l	--	--	27.50-250.00[b]	60.00-150.00[c]	30.00-40.00
Copper mg/l	--	--	0.04-0.40[d]	0.42-1.18[c]	0.14-0.50
Sulfate mg/l	14.00-32.00[c]	0.00-5.00[d]	16.60-23.50[d]	22.00-30.00[c]	5.50-25.50
Temperature °C	16.5-22.00[b]	15.0-22.50[b]	15.50-23.00[b]	16.00-22.50[b]	15.50-23.00

[a] Values represent ranges from July 1 of year indicated to June 30 of the following year.
[b] These values found to be not significantly different from values of following year by Wilcoxon's rank sum test at $p < 0.05$ level.
[c] These values found to be significantly higher than values of following year by Wilcoxon's rank sum test at $p < 0.05$ level.
[d] These values found to be significantly lower than values of following year by Wilcoxon's rank sum test at $p < 0.05$ level.

There were 5 values in 1974 that were higher than 1975: nitrate, total phosphate, chloride, copper, and sulfate. This trend indicates worsening water conditions from 1972 to 1974 with the trend lessening somewhat between 1974 and 1975. Even at best, in 1972, the water quality in the Flat Creek Embayment remained in a severly degraded state.

In Appendix VII, hour one represents 12 o'clock noon, Wednesday, August 22, 1973 when the first 48 hour monitoring period began. Statistical methods have not been used to determine significance, but regular trends may be pointed out. About 1 a.m. Thursday morning, the values for several of the tests indicated changes that suggest the release of effluents into the headwaters of Flat Creek. During this period, chloride levels increased from around 50 mg/l to 300 mg/l, copper levels increased from less than 0.1 mg/l to 0.35 mg/l, nitrate levels went from 5.0 mg/l to 13.0 mg/l, total phosphate levels went from less than 20 mg/l to greater than 40 mg/l, color went from less than 100 APHA units to 195 APHA units, and the pH fell from 8.95 at hour 10 (10 p.m., Wednesday, August 22) to 6.90 at hour 18 (6 a.m., Thursday, August 23).

This trend suggests relase of pollutants during peak daytime sewage usage, with them reaching the sampling site in the early morning hours of Friday. Another consideration in this type of study is the amount of precipitation that occurs. Heavy precipitation began in this study at hour 26 (2 p.m., Thursday, August 23), and continued intermitantly throughout the remainder of the 48 hours. If the pattern of the pollutant levels was indeed caused by the release of industrial or domestic wastes into the water, a reproducable pattern should occur during the second 24 hour period. With the onset of rain, however, changes in test values

could not be directly attributed to industrial and domestic discharges. Though many of the same test values were altered during the rainy period, an equal number of different test values were altered making comparisons of the two cycles meaningless.

Chloride levels did not change appreciably from the onset of rain until the end of the 48 hours. Copper and color both showed increases as the rain started, but tapered off slightly to somewhat lower levels toward the end of the 48 hours. Iron, sulfate, nitrate, and ortho-phosphate showed similar decreasing patterns. The D.O. values also increased, but reached a plateau and did not decline towards the end of the 48 hour period. Values for pH, meta- and total-phosphate, and nitrate remained the same throughout the period that rain fell. Large increases in silica and turbidity values occurred during the rain probably as a result of erosion.

The increase in water flow in Flat Creek which resulted from the heavy rains eventually caused a stabilization of the test values. After changes in the values caused by the initial increased water volume (hours 30 through 40), a plateauing of the test values took place in the last six to eight hours of the monitoring period. None of the test values varied to any great extent but remained constant, the only exceptions being silica and nitrate.

Appendix VIII contains the data from the second 48 hour monitoring trip. This trip took place in 1976 and hour number one represents 1 p.m., Friday, April 4, 1976. Unlike the first 48 hour monitoring, the weather remained clear and no precipitation took place. Chloride tests were not performed in 1976, but hydrogen sulfide and detergent tests were added to the list of water quality parameters examined.

This 48 hour period did not reveal as distinctly the trends seen in the 1974 monitoring effort. A suggestion of the cycle seen in hours ten through twenty of the first 48 hour monitoring can be seen in the second 48 hour period; however, instead of starting around 1 a.m. the following morning, this peak seems to have begun somewhat earlier, about 10 or 11 p.m. Friday night. Five values in the first 48 hour monitoring showed the increase at night following the week day. Excluding chloride (which was not done in the second 48 hour period), 4 of the same remaining tests showed a similar cycle in the second 48 hour period. Copper, nitrate, color, and total-phosphate all demonstrated the same increase around midnight and decrease within eight to ten hours as demonstrated in the 1974 study. The D.O. experienced a slight decrease concomitant with the increase of the other values. The remaining tests, turbidity, iron, pH, nitrite, ortho- and meta-phosphate, H_2S, and detergent, showed no discernable patterns, though it is possible to note unusually aberrant readings for several hours at a time (H_2S readings from hour 1 to 7).

The remainder of the test period showed no easily detectable trends, but this might be expected owing to the fact that this part of the 48 hours fell on a weekend and any possible industrial pollutant sources would not be functioning.

Bacteriological Monitoring

Water samples were collected in the fall of 1973 and the fall of 1975 in the manner outlined in Standard Methods (31) and completed bacterial tests were performed. After gas and acid were produced in the initial lactose broth innoculation, the cultures were then transferred to plates

containing Endo agar. Nucleated colonies with a green metallic sheen developed. Isolated colonies were transferred to agar slants and lactose broths, and again gas and acid were produced. Gram-negative rods with no spores constituted the colonies on the agar slants. The formation of gas in the secondary lactose broth tube and the demonstration of gram-negative nonspore forming rods was considered a satisfactory completed test demonstrating the presence of a member of the coliform group in the sample.

The culture was transferred from the positive primary lactose broth tubes to EC medium. These tubes were then innoculated in a water bath at 44.5°C. Gas was produced within 24 hours, demonstrating a positive reaction and indicating fecal origin. The differentiation of the coliform group into the Escherichia coli, Aerobacter aerogenes, and Escherichia freundii species was performed on the basis of four tests: indole, methyl red, Voges-Proskauer, and Simmons Citrate. In the fall of 1973, the citrate and Voges-Proskauer tests were positive; the indole and methyl red tests were negative. Referring to Standard Methods, the culture on the agar slant was demonstrated to be Aerobacter aerogenes, variety I.

The bacteriological examination conducted in the fall of 1975 again confirmed the presence of Aerobacter aerogenes, variety I. Other colonies were isolated from the agar slants which when subjected to the IMViC reactions gave positive readings on methyl red alone and cultures which gave positive reading on both methyl red and indole. This indicates the presence of both varieties (variety I and variety II) of Escherichia coli. The interperation of these results indicates raw sewage or fecal contamination in the Flat Creek Embayment during the fall of 1975.

CARDIAC RATE STUDIES

Table II contains the data pertaining to the study of the effect of chronic phosphate exposure on cardiac rates in channel catfish. The numbers within the table, except for the last two columns, represent hearbeats per minute. The next to the last column in the table is the arithmetic average of all the rates of the individual fish in each treatment group. The last column is the number of fish in each treatment group. A total of 18 fish were subjected to the first chronic exposure regime in which the phosphate levels were 0, 5, 10, 15, and 20 mg/1. After the 24 hour exposure period at each level, the heart rates were monitored. An immediate increase in heart rate is noted from 115 beats per minute to 130 beats per minute followed by a slight decrease and/or plateauing as the concentration increases up to 20 mg/1. Of the total of 18 fish, 8 of them were maintained on a chronic exposure regime. The cardiac rates of these 8 fish remained elevated and increased slightly after exposure to 20 mg/1 of phosphate for 72 hours and two weeks. As the phosphate level is decreased, however, the cardiac rates also tend to decline and approach a normal control state.

These data suggest that there is a dose response effect of chronic phosphate exposure that is reproducible. The phosphate clearly stimulates cardiac function and metabolism and a slight acclimation takes place as the concentration increases towards 20 mg/1. It is also clear that the cardiac rates remain elevated over extended periods of time. However, no permanent effect of the phosphate is manifested over the time span of this experiment when the fish are withdrawn from the phosphate exposure situation.

Table II

EFFECT OF CHRONIC PHOSPHATE EXPOSURE ON CARDIAC RATE

Fish #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	$\bar{X}$	N
Phosphate (mg/l)																				
0	120	116	132	132	124	116	112	112	96	96	104	112	120	120	100	136	108	112	114.9	18
5[a]	132	128	148	160	128	124	116	140	108	108	124	144	136	140	104	144	121	136	130.1	18
10[a]	140	124	136	148	128	140	124	128	102	102	128	120	144	140	120	144	124	136	129.3	18
15[a]	124	120	128	156	120	140	120	124	104	108	132	120	148	132	128	140	128	136	121.6	18
20[a]	116	108	136	140	112	128	124	116	99	100	132	124	156	144	128	148	132	128	126.2	18
20[b]											124	132	140	128	128	148	129	144	134.0	8
20[c]											134	138	152	140	116	144	132	148	136.8	8
15[a]											128	112	128	141	124	144	136	144	132.1	8
10[a]											128	120	136	120	130	138	128	136	129.5	8
5[a]											120	116	133	120	126	136	96	128	121.9	8
0[a]											110	115	127	120	115	136	104	124	118.9	8

[a]Fish exposed to this and subsequent concentrations for 24 hours

[b]Fish exposed to this concentration for 72 hours

[c]Fish exposed to this concentration for 2 weeks

The effects of acute exposure to phosphate was also examined with the time span being shortened to less than one day. Table III contains the heart rates of fish that were exposed for one hour to phosphate levels that went from 0 to 20 mg/l in two mg/l increments.

It appears that there is not a great deal of difference between the effects of chronic and acute phosphate exposure to catfish; the same trend, with roughly the same rate increase, is demonstrated. The greatest heart rate increases occur somewhat later in Table III than in Table II. However, the same lessening and plateauing of values occurs.

Table IV contains the data resulting from the second acute phosphate exposure regime. In this study, exposure times are still one hour, but the increments of increase are 20 mg/l and the final concentration is 160 mg/l. This final concentration of 160 mg/l is unrealistically high for even a grossly polluted waterway, but it does add insight into the study of physiological effects of phosphates. In Table IV an elevation in heart rate is evident, but after the initial increase there is not a great deal of change over the range of phosphate concentrations. There is a slight decreasing trend to note between 60 and 120 mg/l; however, with the final two values at 140 and 160 mg/l being higher, and all points being statistically different from the controls but not different from one another, little if any significance should be placed in this trend.

The results of these two acute exposure regimes indicates that phosphate causes an increase in cardiac rhythm commencing at levels as low as 2 mg/l and continuing up to 12 to 14 mg/l. Thereafter, cardiac rates plateau at a level somewhat lower than the maximum value and there

Table III

EFFECT OF ACUTE (2 mg/l Increment Increases) PHOSPHATE EXPOSURE ON CARDIAC RATE

Fish #	1	2	3	4	5	6	7	8	$\bar{X}$
Phosphate Concentration mg/l	Heart Beats Per Minute								
0	81	92	88	86	74	86	72	91	83.4
2	82	108	100	98	74	98	73	109	92.8
4	84	108	107	106	82	105	80	112	98.0
6	86	111	114	109	88	108	77	108	100.1
8	88	113	112	112	88	104	76	106	99.9
10	88	110	112	114	90	105	78	108	100/6
12	90	114	110	114	91	108	80	106	101.6
14	86	114	106	112	88	108	80	104	99.8
16	78	105	94	106	77	95	73	92	90.0
18	82	106	94	106	82	104	75	94	92.9
20	78	110	98	112	88	102	78	93	94.9

Table IV

EFFECT OF ACUTE (20 mg/l Increment Increases) PHOSPHATE EXPOSURE ON CARDIAC RATE

Fish #	1	2	3	4	5	6	7	8	$\overline{X}$
Phosphate Concentration mg/l	Heart Beats Per Minute								
0	84	92	76	80	71	86	74	94	82.1
20	100	107	92	91	85	108	88	110	97.6
40	94	103	86	94	96	111	82	106	97.1
60	93	106	86	97	91	105	85	104	95.9
80	90	105	83	99	88	102	82	104	94.1
100	93	102	84	102	87	99	79	108	94.3
120	92	103	84	100	84	96	78	109	93.3
140	92	100	85	108	88	111	84	112	97.5
160	94	103	88	105	86	107	81	109	96.6

is essentially no change in rates up to levels as high as 160 mg/l. The chronic exposure regime also indicates peak values at levels lower than 20 mg/l. However, continued exposure to 20 mg/l for up to two weeks did not cause an accomodation but rather a continued small increase in cardiac rate due to the continued high levels of phosphate. That high levels of phosphate have an effect on cardiac rate in the channel catfish is unequivocally supported under the experimental conditions of this experiment.

Opercular rates were found to be essentially the same as heart rates as indicated in Figure 8. This figure contains selected tracings of both the heart and opercular rhythms and shows that the two are correlated. The cough noted in Figure 8 is a regular phenomenon exhibited by the fish and serves to clear debris from the gill rakers.

An acute exposure regime beginning with 0 mg/l of sulfate and going to 25, 50, 100, 200, 400, 800, 1600, and 3200 mg/l gave the heart rates presented in Table V. The same tank system used in the phosphate experiments was used, and exposure times were one hour. These concentrations are extremely high, and it is doubtful that these sulfate levels, like the extremely high phosphate concentration, would be encountered in even a drastically polluted waterway. Analysis of variance revealed no effect of treatment (the mean square among groups was less than the mean square within the groups), however, there was an unexplainable increase at 1600 and 3200 mg/l of sulfate indicating that sulfate at extremely high levels might become toxic to the catfish.

FIGURE 8

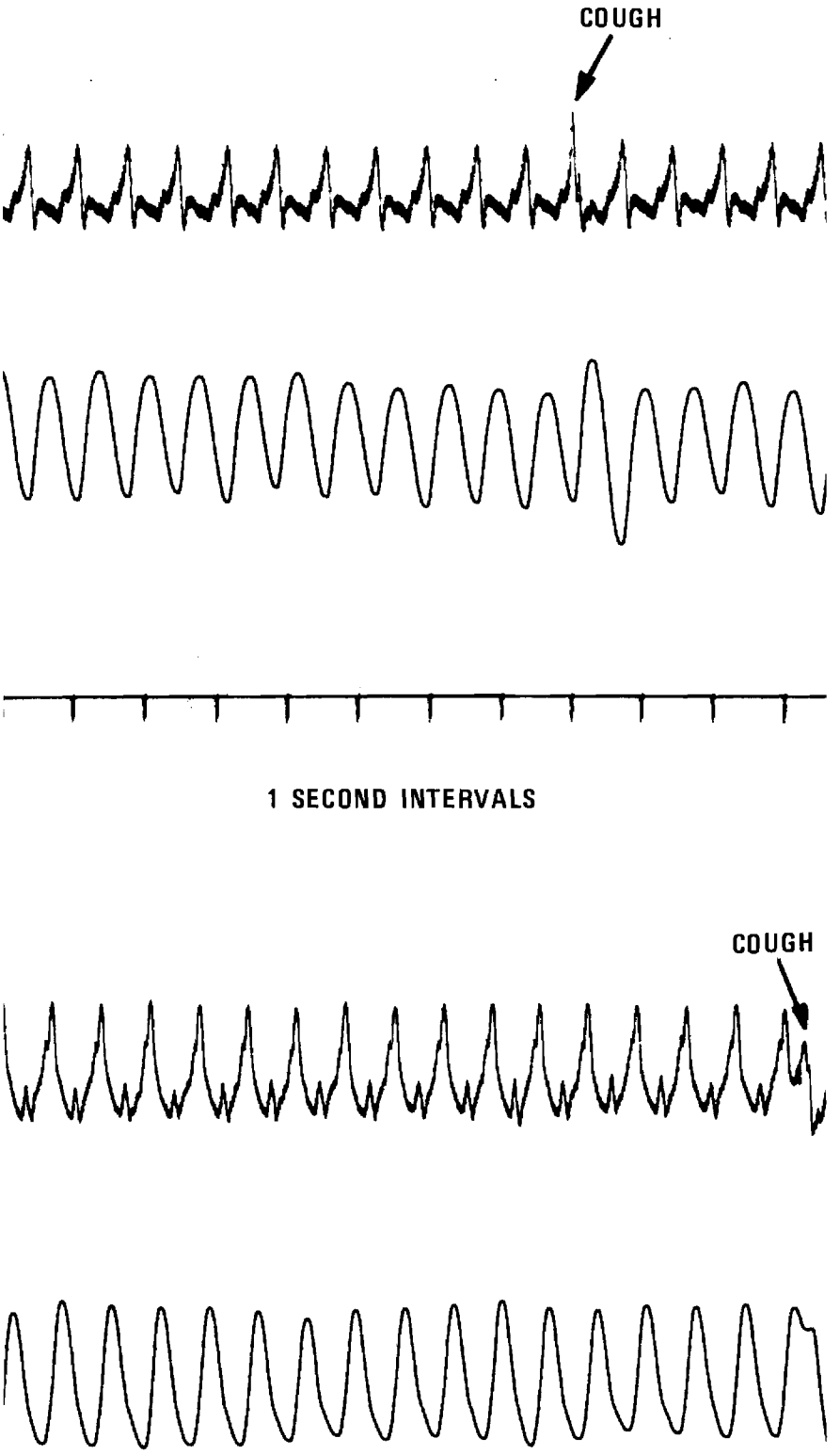


Figure 8. Selected Cardiac and Opercular Rhythm Traces

Table V

EFFECT OF ACUTE SULFATE EXPOSURE ON CARDIAC RATE[a]

Fish #	1	2	3	4	5	$\bar{X}$
Sulfate mg/l	Heart Beats Per Minute					
0	102	96	99	120	96	102.6
25	102	96	102	102	102	100.8
50	102	101	107	114	102	105.2
100	108	96	107	108	96	103.0
200	114	102	102	114	102	106.8
400	114	102	104	114	102	107.2
800	120	90	105	120	90	105.0
1600	126	110	111	126	114	117.4
3200	126	116	114	132	120	121.6

[a]Mean square among groups is less than mean square within groups; therefore no evidence for presence of treatment effect exists using ANOVA

The data in Table V suggest that the ionic character of phosphate is not responsible for the changes in cardiac and opercular rhythms. The design of this experiment did not reveal any toxic or other effect of elevated sulfate levels.

The methodology developed in this project for measuring cardiac and opercular rates served well and afforded a convenient, simple method that was helpful in assessing the toxicity of these substances. It will be possible to use this apparatus in the future to help assess the toxic effects of any substance that is found in aquatic ecosystems.

METABOLIC STUDIES

Effects of Phosphate on Respiration

Table VI has listed in it the oxygen consumption rates for three fish exposed to phosphate levels of 0, 5, 10, 15, and 20 mg/1. Change in percent saturation of the known volume in the test chamber was used to calculate the O_2 consumption of the fish for one hour, corrected to one gram of tissue. Increases in O_2 consumption are noticeable in all three fish at the 5 mg/1 phosphate concentration and continue to increase as the concentration of phosphate goes to 10 mg/1. The rates of consumption then plateau and remain around the same value for the 15 and 20 mg/1 concentration. Temperature remained at 25°C $\pm$ 0.5°C throughout the experimental period and the fish had been allowed several weeks to acclimate to this temperature before experimentation began. Because the solubility of oxygen in water is dependent upon barometric pressure, this value was also recorded each day.

In examining the control values of oxygen consumption, a great deal of variability can be noted. Fish number 2 is the smallest fish, weighing

Table VI

EFFECTS OF PHOSPHATE ON OXYGEN CONSUMPTION

O_2 Consumption--ml/O_2/g tissue/hr.

mg/l PO_4	Fish 1	Fish 2	Fish 3
0	0.101	0.168	0.184
5	0.172	0.216	0.208
10	0.186	0.227	0.226
15	0.235	0.221	0.234
20	0.208	0.227	0.226

105.3 g and Fish 3 is heaviest at 174.0 g. It would be expected that the smaller fish, with a greater surface area to volume ratio would consume more oxygen per gram of tissue than a larger fish would. Fish number 1, which weighed 108.9 g and had the lowest control rate, further reflects the difficulty encountered in attaining a basal state of oxygen consumption.

That phosphate increased metabolic activities in the fish, however, is evident. The responses of all three fish, while starting from different control states, were very similar. Each fish showed an immediate response at 5 mg/l of phosphate and continued to respond in the same fashion as the consumption rates plateaued at roughly the same point. The plateauing effect and the closeness of the consumption values in this region suggests that phosphate causes a very reproducible elevation of oxygen consumption. At the higher levels of phosphate, the metabolic system of the catfish may be stimulated as much as it possibly can be and further exposure may only result in a toxic effect.

Further experimentation with this apparatus might reveal if higher phosphate levels are indeed toxic to the fish. Additionally, more insight into the basal oxygen consumption rates might be gained by letting each fish experience the manipulations required in the experimental procedure several times before beginning the actual experiment. This would reduce the possibility of erroneous results stemming from an excited fish.

Though the question of whether the experimental fish were actually in a basal control state needs elucidation, the effect of phosphate on oxygen consumption has been clearly demonstrated. The experimental design used in this phase of the study was not developed to provide an answer in

exhaustive detail, but to serve as a corollary to the findings in the cardiac rate study. The results indicate both increased basal metabolic rates and increased cardiac functions occurring maximally at a point somewhere between 10 and 20 mg/l.

Food Conversion Efficiency Studies

Results from the food conversion efficiency study appear in Table VII. The mean percent weight increase is the arithmetic average of the 9 weight gain values for the control, 10 mg/l, and 20 mg/l phosphate treatment groups. The initial analysis of variance statistical test performed on the percent weight increase data indicated that the exposure of catfish to phosphate resulted in a significant treatment effect at the $p < 0.05$ level. An *a priori* test was performed subsequently to determine if the control group (no phosphate) was significantly different from the treatment groups (10 and 20 mg/l of phosphate). At the $p < 0.05$ level, the treatment groups were found to be significantly different from the control group.

The mean percent length increase represents the arithmetic average length increase of the 9 fish in each group. Again using analysis of variance, a significant difference between groups was noted as a result of treatment ($p < 0.05$). The *a priori* test between the control group and treatment groups again indicated a significant difference at the $p < 0.05$ level.

The data indicate a definite increase in both weight and length with exposure of the fish to phosphate. In the initial analysis of variance tests, the critical F value was 3.40 in all three metabolic measurements,

Table VII

EFFECTS OF PHOSPHATE ON GROWTH AND FOOD CONVERSION (N = 9)

	Mean % Weight Increase[a]	Mean % Length Increase[a]	Mean Food Conversion Efficiency[b]
Control	15.0	2.6	7.9
10 mg/l PO_4	18.2	4.4	8.1
20 mg/l PO_4	16.2	3.3	7.5

[a]Treatment groups are significantly different from control using analysis of variance at $p < 0.05$ level.

[b]Treatment effect not significant at $p < 0.05$ level using analysis of variance.

and computed F values for the weight and length gains were 46.69 and 51.4, respectively. These values indicate a very marked difference between the groups that would easily withstand even more rigorous statistical testing at the $p < 0.01$ level. The critical value for the a priori tests to determine if the treatment groups were significantly different from the control group was 4.26. Again the calculated F value for the weight and length gains was far greater than the critical value.

While the phosphate treated fish are statistically heavier and longer than control fish, no increase in the conversion efficiency of food to body mass is noted. The calculated F value (2.88) for the percent food conversion efficiency increase is less than the critical F value ($p < 0.05$).

An interesting trend in these data in Table VII is evident. Though this experimental design does not allow adequate insight into the suggestion, a biphasic response of metabolism to phosphate exposure seems to be indicated. At 10 mg/l, all three of these metabolic parameters were elevated above control levels. As the phosphate concentration reached 20 mg/l, all three of these parameters decreased to a level below the 10 mg/l values, but to a level still above the control values. This tends to suggest that in low levels phosphate stimulated growth, but as the concentration increases, there is a more complex mode of action exerted upon the fish. Indeed, high levels of phosphate may both stimulate and depress different physiological parameters. The greatest increase in length and weight was again at a level less than 20 mg/l which parallels the findings in both the cardiac and metabolic studies.

BIOCHEMICAL STUDIES

Effects of Phosphate

The data in Table VIII resulted from the study to determine what effect phosphate had on LDH activity. These data clearly show a pattern similar to that found in the cardiac and respiratory rate experiments. Even though the sample size was small, with only two fish being examined, the trend is still evident. Initially increases occur reaching a peak at 10 mg/l and then declining slightly with a plateau similar to the ones seen previously. At 5 mg/l of phosphate, there was no statistically significant effect, but the data are very consistent with previous findings. If the sample size were to be increased only by one or two fish, then the effect at 5 mg/l and 20 mg/l would be significant.

The study of the effect of phosphate on PyK activity yielded the data presented in Table IX. The sample size in this experiment was larger (four fish were examined), and instead of an increase in activity, there was a pattern of decreasing activity. The only statistically significant decrease occurred at 5 mg/l of phosphate. No significant decrease due to treatment was seen at the other phosphate levels, but a trend is very evident. The reason that these data yielded only one significant change was that the means were computed from values that had more variability than the LDH values did. Elevated phosphate levels in this assay produced results which were reciprocal in nature to all other tests employed in this project.

The results in Table X indicate that statistically, phosphate had no effect on HK activity. Variability of the values used to compute the means again resulted in the failure to detect a treatment effect. Still

Table VIII

EFFECTS OF PHOSPHATE ON
LACTATE DEHYDROGENASE ACTIVITY

Phosphate mg/l	$\overline{X}$ LDH Activity Units/mg Tissue (N = 2)	Significance[a]
0	4.362×10^{-4}	Control
5	6.331×10^{-4}	-
10	15.400×10^{-4}	+
15	13.210×10^{-4}	+
20	11.123×10^{-4}	-

[a]Students t-test, $p < 0.05$ level.

Table IX

EFFECTS OF PHOSPHATE ON PYRUVATE KINASE ACTIVITY

mg/l Phosphate	$\overline{X}$ PyK Activity Units/mg Tissue (N = 4)	Significance[a]
0	8.58×10^{-5}	Control
5	8.64×10^{-6}	+
10	2.22×10^{-5}	-
15	3.78×10^{-5}	-
20	7.35×10^{-5}	-

[a]Student's t-test, $p < 0.05$ level.

Table X

EFFECT OF PHOSPHATE ON HEXOKINASE ACTIVITY

Phosphate mg/l	$\bar{X}$ HK Activity Units/mg Tissue (N = 4)	Significance[a]
0	1.26×10^{-3}	Control
5	2.40×10^{-3}	-
10	3.37×10^{-3}	-
15	5.14×10^{-3}	-
20	2.77×10^{-3}	-

[a]Students t-test, $p < 0.05$ level.

quite noticeable is the type of trend seen in the previous experiments. It is not as evident in Table X, but again, there is an increase to a peak value at approximately 15 mg/l, with a tapering off. Examining higher levels of phosphate exposure might provide insight as to whether a plateauing takes place in the enzyme activity.

The attempts to electrophoretically characterize the LDH isozyme system of the catfish met with only moderate success. Repeated attempts with the different electrophoretic systems suggested four bands. Two major bands, one of which was neutral and did not migrate from the origin and one which migrated slightly toward the anode, were distinct. Two minor bands were also exhibited, but were very faint in the cellulose acetate strips and did not appear at all in the starch gel. One minor band migrated cathodally with slight electrophoretic mobility and the other band moved anodally with greater electrophoretic mobility.

The starch gel system proved to be totally inadequate, and best results were obtained with cellulose acetate.

The two major bands were the only ones trimmed from the cellulose acetate strips, dissolved, and considered in determining the percent activity of each subunit. The minor bands did not always appear in the cellulose acetate strips so their contribution to the total LDH activity, relative to the major bands, was considered negligible. The neutral band was determined to contribute 30.1% of the total activity and the negatively charged band was determined to contribute 69.9%. More work with the cellulose acetate systems using different buffer systems and pH is desirable.

Stresses to an organism result in an additional demand for high energy compounds. Phosphate stress, as employed in this project, has resulted in elevated cardiac rates, respiratory rates, growth rates and biochemical alterations. Increased cardiac function will increase demands for products which can serve as high energy sources. LDH isozyme patterns are very sensitive to changing cardiac function. It is to this end that electrophoretic isolation of LDH isozymes was attempted. Failure to adequately characterize these isozymes completely did not allow for the determination of the effect phosphate had on either the neutral band (probably the M complex) or the negatively charged band (probably the H complex). However, total LDH activity was seen to increase significantly with increasing phosphate levels. Increased LDH levels would be expected in cardiac stressed organisms due to the potential build up of lactate in the heart muscle.

Liver levels of PyK decreased while HK levels increased. The demand for high energy compounds in a stressed organism could possibly lead to the mobilization of glucose and its subsequent phosphorylation by HK. With increased intracellular levels of inorganic phosphate available to those phosphate-stressed fish, the conversion of PEP to pyruvate would be suppressed due to the likelihood of near saturation of ADP and other high energy intermediates.

With the suggestion that the same response patterns take place biochemically as do metabolically, further research is indicated to elucidate the effects phosphate has at a cellular or subcellular level. All indications point to the fact that changes take place in the catfish (and possibly

other organisms) that do not involve just one system. Cardiac, respiratory and growth rates have suggested, in addition to biochemical findings, that the entire fish responds to slightly elevated phosphate levels on an organismal level. No residual effect of exposure was noted during the time periods of the experiments conducted in this project, and either a slight acclimation to the phosphate occurred, or the higher concentrations exerted a possible toxic effect.

Effects of Sulfate

Table XI contains the data resulting from the study of the effect of sulfate on LDH activity. The overbearing observation of these data suggests sulfate has no effect on LDH activity. The t-test, with only one degree of freedom, was not able to detect a statistically significant difference between the control and treatment means.

As with the LDH activities, sulfate treatment did not exert a detectable effect on PyK activity (Table XII). Table XIII contains the results of the study on the effect of sulfate on HK activity. Here again, no effect is noted.

The results in Tables XI, XII and XIII do not support the hypothesis that the ionic nature of phosphate alone is responsible for its effects. Clearly, the sulfate ions do not bring about the profound changes in the catfish that the phosphate ions do. In all of the replications of the experiments in which sulfate was substituted for phosphate, no similar effects were noted. It is doubtful whether considering sulfate in the suggested expanded phosphate experiments would be of any benefit. It is clear there are no parallels between these two ions based on the findings of this report.

Table XI

EFFECT OF SULFATE TREATMENT ON LDH ACTIVITY

Sulfate mg/l	$\bar{X}$ LDH Activity Units/mg Tissue (N = 2)	Significance[a]
0	2.136×10^{-3}	Control
25	2.229×10^{-3}	-
50	2.316×10^{-3}	-
100	2.353×10^{-3}	-
200	2.004×10^{-3}	-
400	3.629×10^{-3}	-
800	3.698×10^{-3}	-
1600	3.900×10^{-3}	-

[a]Students t-test at $p < 0.05$ level.

Table XII

EFFECT OF SULFATE ON PYRUVATE KINASE ACTIVITY

Sulfate mg/l	$\bar{X}$ PyK Activity Units/mg Tissue (N = 2)	Significance[a]
0	1.08×10^{-3}	Control
25	1.96×10^{-3}	-
50	2.86×10^{-3}	-
100	1.95×10^{-3}	-
200	4.76×10^{-3}	-
400	5.25×10^{-3}	-
800	1.60×10^{-3}	-
1600	3.33×10^{-3}	-
3200	4.21×10^{-3}	-

[a]Students t-test, $p < 0.05$ level.

Table XIII

EFFECT OF SULFATE ON HEXOKINASE ACTIVITY

Sulfate mg/l	$\bar{X}$ HK Activity Units/mg Tissue (N = 2)	Significance[a]
0	2.11×10^{-4}	Control
25	3.58×10^{-4}	-
50	2.27×10^{-4}	-
100	1.92×10^{-4}	-
200	1.36×10^{-4}	-
400	4.80×10^{-4}	-
800	2.45×10^{-4}	-
1600	1.70×10^{-4}	-
3200	2.70×10^{-4}	-

[a]Student's t-test, $p < 0.05$ level.

Radiolabelling Experiments

In the first radiolabelling experiment.fish were exposed to 10 mCi $H_3P^{32}O_4$ in 20 liters of water for 1 day, 1 week and 2 weeks. Following exposure, the brain, gill, heart, kidney, liver, muscle (body wall), stomach, and bone (pectoral fin) were analyzed by liquid scintillation to determine to what extent the incorporation of P-32 occurred in these tissues following a direct exposure to phosphate. The data is presented in Table XIV.

The gills of these fish incorporated the largest amount of radiolabelled phosphate. There was no difference between the three time periods. The high levels of phosphate incorporation is due for the most part to the role of the gills. The water is constantly being flushed over the gills and a high level of activity would be expected in this tissue. Levels of radioisotope in the stomach, kidney and heart were also stable during the two week testing period. In the stomach this probably indicates that the phosphate ingested did not accumulate in significant amounts during the course of the experiment although there was an increasing amount of P-32 recovered at each time period. In the kidney the amount was stationary throughout the experiment probably indicating (1) the level at which kidney tissue will incorporate phosphate, and (2) the rate of excretion of this ion. The heart tissue likewise appears stationary when looking at the overall experiment, but showed nearly a 100% increase in labelling at one week when compared to one day, and a subsequent 20% drop from one week to two weeks. The increase at one week is significant ($p < 0.05$) but the decrease at week 2 is not. The other tissues, brain, muscle, liver, and bone, showed significant increases

Table XIV

P-32 LABELLING IN CATFISH FINGERLINGS EXPOSED TO 10mCi $H_3P^{32}O_4$ IN 20 LITERS OF WATER

Day	1	7	14	Comment
Tissue				
Gill	880[a]	780	924	No change
Stomach	141	185	193	No change
Kidney	113	135	124	No change
Heart	45	82[c]	66[c]	Increase 1st week, then steady
Brain	18	36[c]	262[d]	Increases
Muscle	67	134[c]	192[d]	Increases
Liver	79	155[c]	260[d]	Increases
Bone	201	188	449[e]	Steady 1st week, then increases
Water[b]	438	439	407	No change

[a] dpm/g wet tissue

[b] dpm/ml water x 10^3

[c] Paired t-test $p < 0.05$ day 1 vs day 7.

[d] Paired t-test $p < 0.05$ day 7 vs day 14.

[e] Paired t-test $p < 0.05$ day 1 vs day 14.

over the course of the experimental period. The brain tissue increased 100% at one week and over 600% between weeks one and two. The muscle increased 100% between day one and one week and approximately 40% between weeks one and two. The liver increased almost 100% between day one and one week and 67% between weeks one and two. The bone showed no increase (less than 10% decrease) between day one and one week, but nearly a 125% increase at two weeks. While the brain, muscle and liver may represent gradual buildup of phosphate levels due simply to recycling of phospholipids, it might also represent the enhancement of microsomal enzyme systems following an initial priming period. The bone tissue is in the business of manufacturing new bone, particularly in these young fingerlings. The lack of labelling at one week may be artificial or real; however, the values obtained at two weeks probably represent the activity of the osteocytes in their bone-forming capacity.

In the second radiolabelling experiment, Daphnia were exposed to $H_3P^{32}O_4$ as described in the Materials and Methods section. Incorporation of P-32 (Table XV) at 3, 5, 10 and 15 minutes was approximately 157 x 10^3 dpm/mg of Daphnia (dry weight). This represents the initial intake of phosphate into and incorporated on the surface of the Daphnia. At 30 minutes of exposure the radioactivity was measured at 543.8 x 10^3 dpm/mg, an increase of more than 350% from the 15 minute observation. At one hour, the level of activity was 1,324.8 x 10^3 dpm/mg representing a 240% increase over the 30 minute value. At two hours the PO_4 incorporation was 1,550.5 x 10^3 dpm/mg. The next two observation periods, four and eight hours, revealed a decline in the activity recovered, giving 957.4 x 10^3 and 521.0 x 10^3, respectively. The final three observation periods,

Table XV

MEAN P-32 INCORPORATION OF *DAPHNIA*
EXPOSED TO 0.25 mCi FOR VARYING TIME PERIODS

Time	dpm/mg dry wt. x 10^3
3 mins.	153.7[a]
5	156.6[a]
10	164.4[a]
15	154.3[a]
30	543.8[b]
1 hour	1,324.8[c]
2	1,550.5[c]
4	957.4[d]
8	521.0[e]
16	679.0[e]
24	884.5[e]
48	952.4[e]

[a-e]Mean values bearing different superscripts are significantly different (t-test, $p < 0.05$) from the preceding mean.

16, 24, and 48 hours, showed a gradual increase in observed dpm/mg being 679, 884.5 and 952.4, respectively. Statistical analyses of these data suggest that incorporation at 3, 5, 10, and 15 minutes does not significantly differ. Thirty minutes is significantly different from 15 minutes and one hour is significantly different from 30 minutes. One and two hours are not different. Four hours is significantly depressed from two hours, and 8 hours is significantly depressed from four hours. While t-test comparisons between 8 and 16, 16 and 24, and 24 and 48 reveal no statistical differences, the increases from 8 to 24 hours and from 16 to 48 are significant.

The increase in PO_4 incorporation as indicated by the increasing levels of P-32 incorporation up to two hours of exposure is indicative of the initial diffusion of phosphate into and the incorporation of phosphate in the Daphnia organisms. It may also represent the activation of an existing, but not often used, pathway for the conversion and/or storage of phosphate in non-target cells. Still another explanation would be the failure of the Daphnia's transport system to maintain adequate ion balance or for the system to become "overloaded" for a period of time. The third experiment in this series may exclude this as a possibility, however. The fall in recoverable P-32 at the next two observation periods (4 and 8 hours) and the rise in incorporation in the last three periods (16, 24 and 48) is not as easily ascertained. Throughout this experimental period the level of activity in the water ranged from 89 to 152.5 x 10^3 dpm/ml. A possible explanation is that the culture of Daphnia was not pure, containing some small protozoans and numerous bacteria. Also, the rapid generation

time of some of these organisms could result in increased levels of phosphate incorporation. The millipore filtering system utilized (20 microns) would remove most bacteria in the system and their competition for available phosphate would be one possible explanation for lower P-32 levels at 4 and 8 hours. Secondly, the cell may have grown refractory to any additional loading of phosphate and this period may represent normal phosphate removal from the Daphnia. The 24 and 48 hour values are very close and may represent a static situation between the media and the Daphnia. The results from this experiment do not preclude the existance of a diurnal or other cyclic pattern of phosphate incorporation and utilization. Values obtained between 24 and 48 hours and in regular repeating intervals past 48 hours would clarify this point.

It is important from the objectives of this project to note that small crustaceans such as Daphnia are capable of incorporating higher levels of phosphate than they normally carry, and that these high levels of phosphate incorporation had no apparent effect on mortality throughout the duration of the 48 hour experiment.

The third radiolabelling experiment as described in the Materials and Methods involved the labelling of Daphnia with P-32 and then allowing these crustaceans to serve as the sole source of live food for a tank of fingerling catfish. The catfish were sacrificed and various tissues were removed and counted as described previously. Results of this experiment are presented in Table XVI and Figure 9. In an effort to elucidate the meaning of this data, Tables XVII and XVIII are presented. In Table XVII eight Duncan Multiple Range Tests are presented (one for each

Table XVI

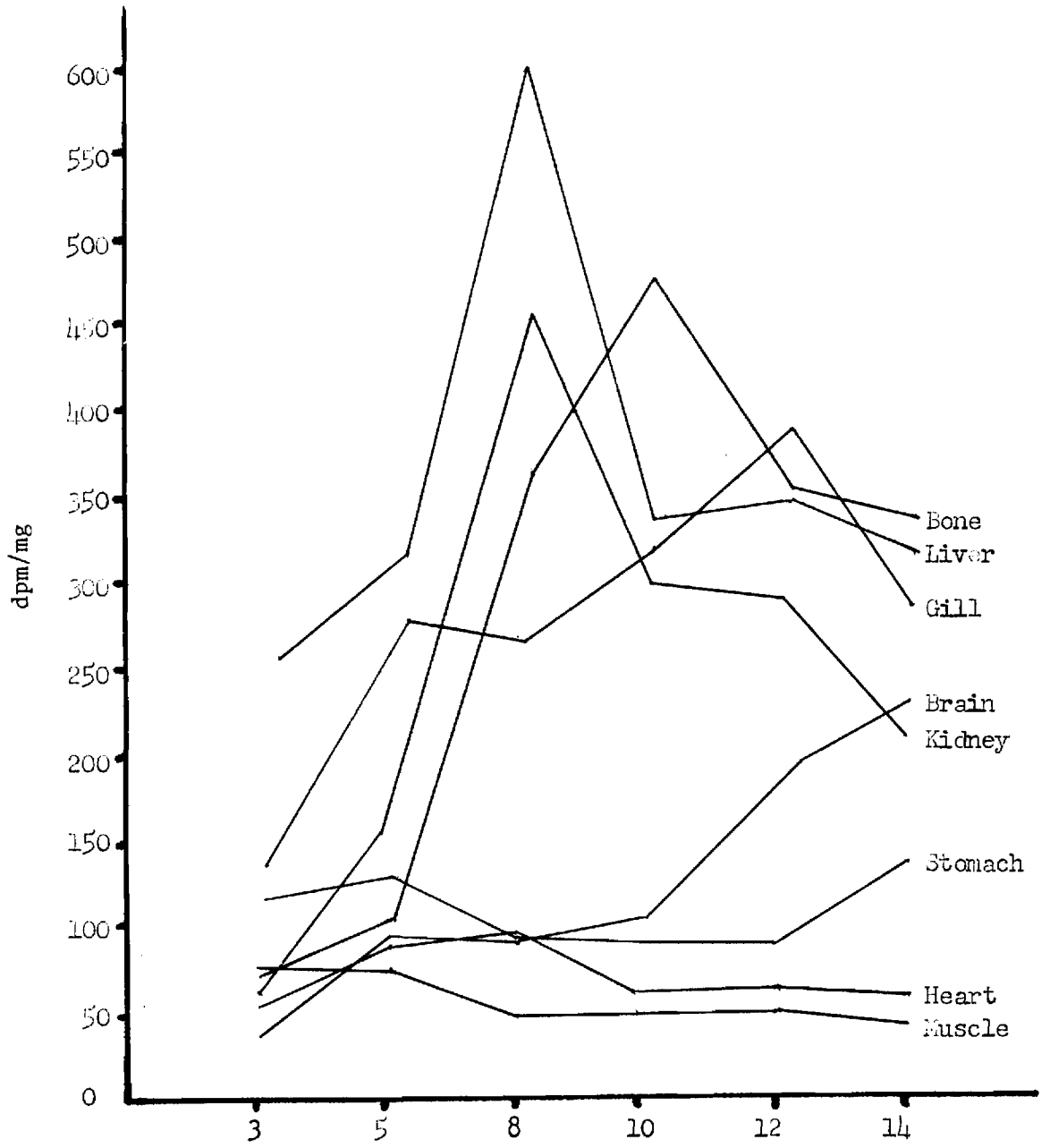
AVERAGE dpm/mg WET WEIGHT OF TISSUE (5 ANIMALS PER TIME GROUP--3 REPLICATES EACH ANIMAL) IN FINGERLING CHANNEL CATFISH FED EXCLUSIVELY ON A DIET OF DAPHNIA REARED IN WATER CONTAINING P-32

Time	Liver	Kidney	Gill	Bone	Brain	Stomach	Heart	Muscle
3 days[a]	255.3	57.1	136.2	72.7	40.0	115.7	55.9	75.2
5 days	316.5	164.7	282.5	109.4	97.5	132.0	89.2	79.2
8 days	606.2	453.5	267.0	366.9	87.6	89.9	94.3	50.4
10 days	337.7	303.3	361.4	483.4	105.1	87.4	62.3	51.1
12 days	348.5	294.1	390.2	358.4	195.9	87.2	64.6	52.6
14 days	304.5	200.2	279.6	339.9	229.9	145.4	61.5	41.4

[a]Catfish fed on Daphnia maintained in culture containing P-32. See text for complete description.

Figure 9

Tissue Levels of P-32 Found in Fingerling Channel Catfish Following Ingestion of P-32 Exposed Daphnia



Number Days Fed P-32 Daphnia

Table XVII

DUNCAN'S MULTIPLE RANGE TEST FOR COMPARISON OF MEAN dpm/mg WET TISSUE AT THE SIX SAMPLING PERIODS[a]

3 days

Liver	Gill	Stomach	Muscle	Bone	Kidney	Heart	Brain
255.3	136.2	115.7	75.2	72.7	57.1	55.9	40.0

5 days

Liver	Gill	Kidney	Stomach	Bone	Brain	Heart	Muscle
316.5	282.5	164.7	132.0	109.4	97.5	89.2	79.2

8 days

Liver	Kidney	Bone	Gill	Heart	Stomach	Brain	Muscle
606.2	453.5	366.9	267.0	94.3	89.9	87.6	50.4

[a]Means underscored by the same line are not significantly different at the $p < 0.05$ level.

Table XVII (continued)[a]

10 days

Bone	Gill	Liver	Kidney	Brain	Stomach	Heart	Muscle
483.4	361.4	337.7	303.3	105.1	87.4	62.3	51.1

12 days

Gill	Bone	Liver	Kidney	Brain	Stomach	Heart	Muscle
390.2	358.4	348.5	294.1	195.9	87.2	64.6	52.6

14 days

Bone	Liver	Gill	Brain	Kidney	Stomach	Heart	Muscle
339.9	304.5	279.6	229.9	200.2	145.4	61.5	41.4

[a]Means underscored by the same line are not significantly different at the $p < 0.05$ level.

Table XVIII

RANKING OF THE SAMPLING MEANS OF THE FINGERLING CATFISH TISSUE TAKEN FROM ANIMALS EXPOSED TO P-32 DAPHNIA

	Liver	Gill	Stomach	Muscle	Bone	Kidney	Heart	Brain
Time								
3 days	1	2	3	4	5	6	7	8
5 days	1	2	4	8	5	3	7	6
8 days	1	4	6	8	3	2	5	7
10 days	3	2	6	8	1	4	7	5
12 days	3	1	6	8	2	4	7	5
14 days	2	3	6	8	1	5	7	4
$\overline{X}$	1.833	2.333	5.167	7.333	2.667	4.000	6.667	5.833
	Liver	Gill	Bone	Kidney	Stomach	Brain	Heart	Muscle
$\overline{X}$ Rank	1.833	1.333	2.667	4.000	5.167	5.833	6.667	7.333

sampling period) demonstrating which tissue incorporated significantly greater amounts of P-32 after ingestion of the radiolabelled Daphnia. It is worthy to note that the liver incorporates large quantities of P-32 throughout this experiment and has the highest level of incorporation in three of the six periods sampled. Of the eight tissues sampled, the brain had the least incorporation of P-32 on day three, but showed a steady increase throughout the experiment, ranking fourth after 14 days. Likewise, the bone (dorsal spine) started in fifth position and ranked either first or second during the last three sampling periods. Table XVIII summarizes the ranking of these eight tissues and shows a mean rank for each tissue for the entire experimental period. In decreasing order the tissue ranked liver, gill, bone, kidney, stomach, brain, heart and muscle for the overall experiment. Heart and muscle tissue ranked low consistently throughout the experiment. Values for these two tissues and for the stomach were not significantly different (t-test) from the values obtained at the first sampling period (3 days) and the last (14 days). All other tissues showed a significant increase between day 3 and 14.

The interpretation of these results is fairly clear. The first observation is that when P-32 labelled Daphnia serve as the sole source of food for fingerling catfish, following the digestion of these crustaceans, the P-32 incorporated in these bodies is released into the blood or lymph systems of the catfish. Once in these two transport systems, the P-32 labelled material has the potential to reach any tissue. Lipid bound, nucleoside bound, low molecular weight protein bound, and free inorganic P-32 all eventually find their way to the liver either via the

hepatic portal system or the lymphatic-circulatory systems. The lipid-bound P-32 labelled structures may be removed by other tissues while in the circulatory system or metabolized, but most are eventually returned to the liver. The liver has enzymatic pathways to alter the P-32 containing lipids, store them, or to release them into the circulatory system. Other P-32 containing compounds can likewise be altered, stored, or passed through the liver to be released into the circulatory system. Considering all these activities of the liver, it is entirely possible to find large amounts of P-32 present there throughout the experiment. The gills are subjected to P-32 released by both the Daphnia and by the catfish. The gills can also serve as a point of excretion of ions. It is interesting to note that the gills in this experiment did not exhibit the highest activity of all organs as they did in the catfish labelling experiment. If there were a way to remove the P-32 released by both the Daphnia and catfish into the aquaria, the level of P-32 present in the gills would be considerably lower. A large portion of the label present in this study must also be the result of P-32 in the water.

The bone tissue shows an interesting trend. These are young, rapidly growing fingerlings. Incorporation of P-32 metabolites into the bone displays an active sequestering of phosphate products for the bones by these catfish. It might also be suggested that elevated levels of phosphate, in conjunction with adequate levels of calcium, which is prevalent in the Southeast, might enhance the growth and ossification of the skeletal system.

As more phosphate becomes available to the organism than it can use, the kidney would be one source of excretion. Levels of P-32 in the kidney

represent both the active excretion of the ion and its normal incorporation and replacement of phosphate into the extracellular fluid of the nephron and into the high energy products which are so important to the active transport systems in the kidney.

Levels in the brain increase slowly throughout the experiment. This probably represents phosphate turnover in membrane-bound phosphate and increased myelinization of the central nervous system during this period.

The stomach levels are relatively constant throughout the experiment. These levels represent the level of P-32 incorporation in the stomach tissues in membrane structures and high energy reserves. Levels of P-32 in the stomach could also be influenced by the period of time since the fish last ate. The stomach appears to have little or no capacity for incorporation of luxury amounts of phosphate.

The heart and muscle tissue levels do not change during the course of this experiment. The heart is capable of direct removal of phosphate containing lipids from the circulatory system. However, the heart stores very little in the way of high energy products. For this reason, the level of phosphate in the heart is relatively constant. Similarly, the muscle tissue has only limited amounts of high energy storage capacity. Since lipid stores were not tested in this experiment, there is no comparison of the phosphate levels presented. Given the results seen in the other tissues, lipid levels would probably be expected to increase in an experimental design such as this.

CHAPTER 4. CONCLUSIONS AND RECOMMENDATIONS

For the past five years this investigator has been in close contact with the Flat Creek Embayment of Lake Sidney Lanier. At times, navigation in Flat Creek was impossible due to debris (tires, bottles, cans, trees, and assorted trash). Generally, the influx of large debris into the creek has diminshed over this five year period. This has been helped in part by the installation of a surface log boom over the creek to trap all large objects. The water quality in Flat Creek, however, has not improved significantly in the last three years. The fact that it has maintained its present water quality conditions is indeed encouraging. There does appear to be a diurnal cycling of some of the nutrients present, due principally to the loading of Flat Creek by industrial wastes and by the municipal treatment plant with incompletely treated sewage. An increase in coliforms, as well as a diversification of species, has been observed during the past three years. There continues to be significant amounts of anaerobic bottom activity occuring near the sampling site. Water quality in the Lake Lanier area which is used for water sports (swimming and skiing), some 2000 meters below the sampling site, appears acceptable. Heavy alga and protozoan blooms still occur in Lake Lanier below the Flat Creek and Balus Creek outfalls. These studies have been reported by Ahearn and his co-workers (3,4). The loading of Lake Lanier with the nutrients from Flat Creek will continue to intensify and to hasten the advancement of eutrophrication of Lake Lanier.

This study resulted in the design of a cardiac and opercular rate monitoring apparatus utilizing umimplanted electrodes. This process was

heretofore not described in the literature. The monitoring of cardiac function was simplified and made easily replicable by this process. In this study, increases in cardiac function were in response to the elevation of phosphate up to approximately 15-20 mg/l. Increases above 20 mg/l (up to 160 mg/l) failed to increase cardiac function furhter. Indeed, cardiac function plateaued and showed a slight decrease at higher levels. Acute and chronic exposures essentially revealed a gradual increase in cardiac rate; however, when these fish were returned to water without phosphate, their cardiac rates returned to normal.

Basal metabolic activity in the channel catfish was monitored and found to increase with increases in the level of phosphate in the water. Highest values were obtained between 10 and 20 mg/l.

Food conversion efficiency was not altered in fish which were exposed to high levels of phosphate; however, they did consume more food and increase in length and weight at significantly greater rates than control catfish. Again the 10 mg/l fish increased faster than the 20 mg/l fish.

Biochemical studies in which the selected enzyme activities were monitored after fish were exposed to phosphate and sulfate yielded variable results. Heart tissues were analyzed for LDH activity after the fish were exposed to both phosphate and sulfate. This enzyme demonstrated an increase in activity with the phosphate treatment showing a peak around the 10 mg/l phosphate exposure level. Activities then dropped slightly and plateaued at the higher concentrations. The LDH enzyme system showed no change in activity in response to elevated sulfate levels. The HK and PyK studies were conducted on liver tissue. HK activity also demonstrated an increase

in activity, but it was not as clearly evident as the increase in LDH activity. No change in HK activity as a result of sulfate exposure was noted.

PyK activity also did not change as a result of sulfate exposure, but demonstrated a decline in activity as a result of phosphate exposure. The decrease in PyK activity, as with the HK increase, was not as great as the LDH increase.

Phosphate was determined to alter all three of these enzymes, but sulfate did not. This tends to suggest that the ionic nature alone of phosphate is not responsible for the changes it causes in the channel catfish.

Complete electrophoretic resolution of the LDH isozyme system was not satisfactorily achieved in this project. Starch gel systems proved totally inadequate, but cellulose acetate strips did allow for the separation of two major and two minor bands. Further work on the separation of LDH isozymes and their relation to cardiac stress in the channel catfish is suggested.

Radiolabelling experiments revealed the direct incorporation of P-32 phosphate into Daphnia and the channel catfish. All tissues incorporated phosphate at varying amounts with a peak activity occurring within 2 hours in the Daphnia and continued increases in activity in the catfish over a two week experimental period. Catfish which were fed P-32 labelled Daphnia incorporated phosphate in a slightly different pattern than those which were exposed to the phosphate directly through the water. The liver sequestered proportionally more phosphate in the feeding experiment than in the direct exposure experiment.

Studies (cardiac rate, biochemical and radiolabelling) with the sulfate ion failed to duplicate the findings that resulted from the phosphate investigations. This tends to discount the effect of the divalent nature of the phosphate ion being totally responsible for the results obtained in this study.

Further study is indicated in the biochemical area. The elucidation of the LDH isozymes in the channel catfish is of interest to this investigation. Further, to determine if stress or sublethal toxic conditions might be detected by such a biochemical test is a challenge to be met in the near future. The procedure developed in the catfish for monitoring cardiac and opercular rates is expandable to most freshwater fish species and is relatively easy and inexpensive monitor of environmental stress. This process has wide utility, and with proper directives from either OWRT or EPA, a protocol for the testing of possible aquatic toxicants can be pursued.

The basal metabolic chamber which was built for this experiment could be modified for any aquatic organism and would provide another quick and inexpensive measure of possible aquatic toxicants. This measures metabolic activity directly, whereas the cardiac function measures only one parameter.

As the completion report for project A-035-GA(2) reported, algae have the capability of incorporating luxury amounts of phosphates. Apparently, crustaceans such as Daphnia and vertebrates such as the channel catfish can incorporate luxury amounts into their tissues. For the catfish, it was demonstrated that phosphate can have an influence both directly (through the water) and indirectly (through its food source). The possibility of

biomagnification of phosphate, and its possible consequences, need elucidation. Questions which need to be answered are: (1) do high levels of phosphate alter metabolic pathways which might have profound or lasting effects on the organism; (2) do high levels of phosphate become toxic after an extended period of time; (3) do high levels of phosphate encourage storage of phosphates as high energy stores or high energy precursors, (4) of what lasting affect does increased metabolic rates, growth rates, and cardiac rates have on these aquatic organisms exposed to these high levels of phosphate in their everyday environment. Finally, the water quality in Flat Creek, and indeed the entire Lake Lanier area, needs to be continually monitored. With this water system serving so many for recreation and so many as an eventual water supply, attempts at improving the water quality must be continued.

Appendix I

LDH Stain for Electrophoresis

	Reagent	Concentration	Volume
1.	Tris-HCL buffer	0.2M pH 8.0	45 ml
2.	Sodium lactate	0.5M pH 7.0	9 ml
3.	Nitroblue tetrazoleum	1 mg/ml	5 ml
4.	Phenazine methosulfate	1.6 mg/ml	5 ml
5.	NAD^+	10 mg/ml	2 ml

Appendix II

Buffer Dilution for LDH Electrophoresis

Buffer System	Gel Dilution	Anode Dilution	Cathode Dilution
edtate-borate-tris	1:20	1:6	1:4
tris-citrate	1:15	none	none
tris-verseve-borate	1:10	none	none

Appendix III

Equation Used to Calculate Enzyme Activity

$$\text{units enzyme/ml} = \frac{\frac{\Delta OD}{min} \text{ at } 340mm \times 3 \times \text{dilution factor}}{6.22}$$

Appendix IV

Reagents Added to Spectrophometer Reference Cell in LDH Assay

	Reagent	Concentration	Volume
1.	Sodium phosphate buffer	0.1M pH 7.0	2.0 ml
2.	Sodium pyruvate	0.02M	0.05 ml
3.	$NADP^{+}+H^{+}$	10 mg/ml	0.02 ml

Appendix V

Reagents Added to Spectrophotometer Reference Cell in PyK Assay

	Reagent	Concentration	Volume
1.	Imidazole buffer	20 mg/ml	2.6 m/1
2.	ADP	10 mg/ml	0.1 ml
3.	PEP	5 mg/ml	0.1 ml
4.	$NADH^{+}{}_{+H}{}^{+}$	10 mg/ml	0.1 ml
5.	LDH	15 u/μℓ	0.01 ml

Appendix VI

Reagents Added to Spectrophotometer Reference Cell in HK Assay

	Reagent	Concentration	Volume
1.	Triethanolamine buffer pH 7.6	0.1M	1.1 ml
2.	β-D-glucose	10% w/v	1.2 ml
3.	$MgCl_2$	0.1M	0.2 ml
4.	ATP	0.016M	0.1 ml
5.	$NADP^+$	0.013M	0.2 ml
6.	G-6-P dehydrogenase	15 u/µl	0.1 ml

Appendix VII

TEST VALUES FOR FIRST 48 HOUR MONITORING

Hour	1	2	3	4	5	6	7	8	9	10
Chloride mg/l	99	96	104	100	73	68	65	72	60	37
Copper mg/l	0.03	0.03	0.05	0.06	0.06	0.07	0.06	0.07	0.05	0.09
Color APHA units	67	60	75	86	77	85	110	165	180	205
Turbidity FTU	40	42	41	44	42	43	43	43	37	39
Iron mg/l	0.66	0.66	0.69	0.70	0.69	0.68	0.69	0.74	0.76	0.77
Silica mg/l	36.00	34.75	34.00	33.75	3.100	33.00	33.00	32.50	31.50	32.50
Sulfate mg/l	18	22	26	30	28	20	26	29	44	50
pH	7.05	7.00	7.15	8.00	8.00	8.50	8.70	8.90	8.90	8.95
Nitrites mg/l	0.38	0.38	0.42	0.40	0.30	0.21	0.19	0.170	0.19	0.15
Nitrates mg/l	6.50	7.50	7.50	7.00	6.00	6.50	5.00	6.00	4.50	5.00
Phosphate Ortho mg/l	3.70	3.60	3.20	3.10	3.70	3.20	6.00	10.00	5.75	11.00
Meta	8.30	5.40	21.80	8.90	6.50	20.30	8.00	8.00	23.75	21.50
Total	12.00	9.00	25.00	11.00	10.20	23.50	14.00	18.00	29.50	32.50
D.O. mg/l	1.70	1.90	2.10	2.00	2.30	2.00	2.50	4.00	4.00	4.50

Appendix VII (continued)

TEST VALUES FOR FIRST 48 HOUR MONITORING

Hour	11	12	13	14	15	16	17	18	19	20
Chloride mg/l	46	79	110	260	300	195	210	145	150	190
Copper mg/l	0.08	0.10	0.16	0.21	0.35	0.29	0.36	0.30	0.35	0.48
Color APHA units	190	190	170	190	145	135	130	120	135	90
Turbidity FTU	43	40	40	43	36	40	38	39	41	43
Iron mg/l	0.77	0.74	0.65	0.58	0.60	0.52	0.56	0.60	0.56	0.54
Silica mg/l	33.00	33.00	39.00	42.00	45.75	47.00	38.00	42.00	36.75	42.75
Sulfate mg/l	51	49	36	39	28	16	26	26	24	22
pH	8.50	7.90	8.40	8.00	7.60	7.20	7.20	6.90	7.00	7.50
Nitrites mg/l	0.10	0.15	0.12	0.10	0.15	0.05	0.02	0.01	0.02	0.02
Nitrates mg/l	5.50	5.00	6.50	6.50	7.50	7.00	10.50	12.50	13.00	10.80
Phosphate Ortho mg/l	6.20	6.80	6.20	6.00	6.40	7.00	6.20	5.60	6.40	5.20
Meta	9.30	13.20	9.30	14.00	31.10	30.50	35.80	21.90	19.10	16.80
Total	15.50	20.00	15.50	20.00	37.50	37.50	42.00	27.50	25.50	22.00
D.O. mg/l	4.00	4.00	4.50	3.00	2.00	2.00	2.50	3.50	3.50	4.00

Appendix VII (continued)

TEST VALUES FOR FIRST 48 HOUR MONITORING

Hour	21	22	23	24	25	26	27	28	29	30
Chloride mg/l	165	110	95	60	50	45	40	50	40	65
Copper mg/l	0.40	0.40	0.38	0.26	0.20	0.13	0.17	0.12	0.20	0.15
Color APHA units	75	65	90	60	60	55	100	120	180	400
Turbidity FTU	41	44	36	38	30	28	38	85	100	110
Iron mg/l	0.71	0.69	0.36	0.74	1.01	1.23	1.58	1.60	1.59	1.73
Silica mg/l	29.00	23.75	30.00	36.25	39.25	40.25	36.25	41.00	40.30	36.25
Sulfate mg/l	16	19	19	16	15	14	15	9	17	20
pH	7.40	7.30	7.10	7.10	7.40	7.30	7.40	7.20	7.40	7.00
Nitrites mg/l	0.01	0.02	0.01	0.01	0.01	0.01	0.005	0.02	0.02	0.01
Nitrates mg/l	12.20	11.70	11.00	10.50	10.50	9.00	10.00	8.50	9.50	10.00
Phosphate Ortho mg/l	5.00	5.60	8.50	6.30	7.30	10.00	11.00	9.60	18.00	13.50
Meta	19.50	17.40	11.50	10.70	9.70	5.50	5.00	5.90	8.00	2.50
Total	24.50	23.00	22.00	17.00	17.00	15.50	16.00	15.50	26.00	16.00
D.O. mg/l	4.00	4.50	3.00	2.00	1.00	1.50	0.50	1.00	1.00	1.00

Appendix VII (continued)

TEST VALUES FOR FIRST 48 HOUR MONITORING

Hour	31	32	33	34	35	36	37	38	39
Chloride mg/l	60	50	55	40	40	50	37	39	40
Copper mg/l	0.13	0.16	0.15	0.15	0.14	0.22	0.28	0.42	0.43
Color APHA units	420	360	320	220	130	135	130	135	140
Turbidity FTU	105	110	110	65	25	47	35	25	30
Iron mg/l	1.85	2.00	2.30	2.20	1.80	1.60	1.40	1.30	1.10
Silica mg/l	36.50	39.00	200.00	223.25	300.00	375.00	390.00	450.00	340.00
Sulfate mg/l	24	23	26	26	23	27	29	30	27
pH	7.50	7.90	7.60	7.35	7.30	7.30	7.50	7.50	7.60
Nitrites mg/l	0.02	0.01	0.07	0.10	0.13	0.13	0.11	0.12	0.11
Nitrates mg/l	9.50	9.70	9.70	10.00	10.20	10.50	10.50	9.30	8.60
Phosphate Ortho mg/l	8.00	8.00	15.20	15.00	12.40	9.20	11.00	8.80	10.00
Meta	9.00	6.00	1.80	0.00	3.60	8.80	6.00	7.20	7.00
Total	17.00	14.00	17.00	15.00	16.00	18.00	17.00	16.00	17.00
D.O. mg/l	1.50	2.00	3.50	3.50	3.60	3.50	3.50	3.70	3.60

Appendix VII (continued)

TEST VALUES FOR FIRST 48 HOUR MONITORING

Hour	40	41	42	43	44	45	46	47	48
Chloride mg/l	40	41	40	40	39	38	38	38	38
Copper mg/l	0.41	0.36	0.39	0.39	0.38	0.37	0.37	0.36	0.36
Color APHA units	125	130	130	130	125	120	130	120	125
Turbidity FTU	25	20	25	31	36	41	47	47	43
Iron mg/l	1.10	1.20	1.10	1.10	1.00	1.10	1.10	1.10	1.00
Silica mg/l	290.00	460.00	510.00	520.00	580.00	620.00	380.00	360.00	290.00
Sulfate mg/l	26	27	28	28	23	21	23	22	21
pH	7.90	7.70	7.90	7.60	7.50	7.30	7.30	7.20	7.30
Nitrites mg/l	0.13	0.11	0.10	0.10	0.11	0.10	0.10	0.10	0.11
Nitrates mg/l	9.20	7.30	10.50	10.50	10.10	9.30	8.10	3.50	3.50
Phosphate Ortho mg/l	13.00	16.00	15.00	15.00	16.00	15.00	15.50	14.00	15.00
Meta	1.50	2.00	1.00	1.00	0.00	3.50	2.50	2.00	1.00
Total	14.50	18.00	16.00	16.00	16.00	18.50	18.00	16.00	16.00
D.O. mg/l	3.60	3.40	3.50	3.50	3.60	3.60	3.60	3.50	3.60

Appendix VIII

TEST VALUES FOR SECOND 48 HOUR MONITORING

HOUR	1	2	3	4	5	6	7	8	9	10	11
Copper mg/l	0.00	0.20	0.10	0.10	0.15	0.15	0.05	0.20	0.25	0.30	0.25
Color APHA Units	50	60	55	60	30	40	20	90	125	105	80
Turbidity FTU	15.0	17	45	85	22	8	100	35	15	18	30
Iron mg/l	0.55	0.26	0.45	0.33	0.70	0.15	0.35	0.40	0.35	0.90	0.90
Silica mg/l	19	25	27	19	26	25	27	19	14	19	14
Sulfate mg/l	22.0	14	18	16	14	17	18	17	18	16	15
pH	6.69	6.98	6.90	6.80	6.68	6.70	6.83	6.75	6.54	6.40	6.69
Nitrates mg/l	6.0	12.0	13.0	12.0	10.0	10.5	20	38	22	31	33
Nitrites mg/l	0.03	0.00	0.00	0.00	0.00	1.36	0.03	0.56	0.02	0.00	0.14
Phosphates Ortho mg/l	6.0	4.0	4.0	7.0	6.0	8.0	8.0	10.0	9.0	8.0	9
Meta	5.0	3.0	3.0	1.0	3.0	1.0	20	4.0	2.0	4.0	2
Total	7.0	7.0	7.0	8.0	9.0	9.0	10.0	14.0	14.0	12.0	14
D.O. mg/l	-	-	-	9.0	9.0	9.0	8.0	6.0	7.0	6.0	6.5
H_2S	8.0	6.0	0.0	6.0	6.0	6.0	2.0	0.0	0.0	0.0	6.0
Detergent	0.25	0.0	0.3	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0

Appendix VIII (continued)

TEST VALUES FOR SECOND 48 HOUR MONITORING

HOUR	12	13	14	15	16	17	18	19	20	21	22
Copper mg/l	0.30	0.15	.30	0.16	0.05	0.10	0.15	0.05	0.05	0.00	0.15
Color APHA Units	105	90	70	80	75	40	50	55	60	40	80
Turbidity FTU	15	27	20	15	50	55	70	60	65	40	55
Iron mg/l	0.4	0.2	0.8	0.1	0.2	0.5	0.4	0.4	0.1	0.05	0.20
Silica mg/l	19	18	15	16	140	64	91	45	60	65	75
Sulfate mg/l	15	16	17	13	10	12	10	11	10	4	7
pH	6.77	6.77	6.72	6.77	6.40	6.48	6.68	6.78	6.82	6.90	6.81
Nitrates mg/l	33	84	22	32	42	18	15	21	26	26	11
Nitrites mg/l	0.01	0.76	0.08	0.01	0.40	0.26	0.11	0.36	0.20	0.34	0.00
Phosphates Ortho mg/l	12	11	10	11	8	9	8	9	13	8	10
Meta	2	1	1	1	4	0	0	1	0	1	0
Total	14	12	12	12	12	9	8	10	13	9	10
D.O. mg/l	10.0	10.5	11.5	12.5	12.0	14.0	12.0	12.0	14.0	14.5	14.5
H_2S	0.0	0.0	3.0	0.0	0.1	1.0	0.0	0.4	0.0	3.0	1.0
Detergent	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix VIII (continued)

TEST VALUES FOR SECOND 48 HOUR MONITORING

HOUR	23	24	25	26	27	28	29	30	31	32	33
Copper mg/l	0.10	0.10	0.15	0.15	0.10	0.10	0.05	0.10	0.10	0.05	0.10
Color APHA Units	60	30	40	40	40	35	20	40	30	50	20
Turbidity FTU	40	20	15	30	30	20	10	10	20	90	10
Iron mg/l	0.45	0.30	0.10	0.05	0.2	0.5	0.3	0.8	0.40	0.60	0.10
Silica mg/l	90	24	11	13	12	13	10	13	13	12	13
Sulfate mg/l	10	20	12	7	12	3	5	16	5	12	10
pH	6.90	6.96	6.97	6.99	7.01	7.03	6.99	6.97	6.95	6.32	6.33
Nitrates mg/l	18	17	20	16	18	15	13	20	17	12	15
Nitrites mg/l	0.18	0.07	0.24	0.30	0.34	0.26	0.04	0.30	0.05	0.24	0.12
Phosphates Ortho mg/l	9	9	8	7	7	5	7	5	5	8	5
Meta	1	0	2	2	1	2	3	2	3	2	2
Total	10	9	10	9	8	7	10	7	8	10	7
D.O. mg/l	15.0	15.5	16.5	15.0	16.0	15.0	15.0	13.0	12.0	9.0	9.0
H_2S	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0
Detergent	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0

Appendix VIII (continued)

TEST VALUES FOR SECOND 48 HOUR MONITORING

HOUR	34	35	36	37	38	39	40	41	42	43	44
Copper mg/l	0.05	0.10	0.05	0.15	0.15	0.10	0.15	0.10	0.15	0.20	0.20
Color APHA Units	40	40	55	15	40	45	50	50	40	60	45
Turbidity FTU	20	40	90	50	110	90	50	110	30	40	40
Iron mg/l	0.20	0.00	0.10	0.35	0.65	0.35	0.5	0.40	0.40	0.20	0.30
Silica mg/l	14	11	10	12	12	11	10	10	10	11	12
Sulfate mg/l	8	15	3	8	8	7	8	9	11	7	4
pH	6.38	6.52	6.65	6.69	6.73	6.82	6.84	6.86	6.92	6.98	7.36
Nitrates mg/l	35	34	32	31	33	31	34	34	31	33	29
Nitrites mg/l	0.44	0.38	0.44	0.05	0.48	0.52	0.40	0.36	0.34	0.26	0.26
Phosphates Ortho mg/l	5	7	5	5	6	6	8	5	6	4	4
Meta	1	3	2	3	3	3	1	3	2	5	2
Total	6	10	7	8	9	9	9	8	8	9	6
D.O. mg/l	14.0	14.0	12.0	8.0	15.0	12.0	14.0	12.0	16.0	16.0	16.0
H_2S	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Detergent	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix VIII (continued)

TEST VALUES FOR SECOND 48 HOUR MONITORING

HOUR		45	46	47	48
Copper mg/l		0.15	0.10	0.10	0.20
Color APHA Units		40	35	40	40
Turbidity FTU		25	100	125	220
Iron mg/l		0.50	0.25	0.35	0.30
Silica mg/l		8	10	8	10
Sulfate mg/l		5	7	4	10
pH		7.26	7.24	7.24	7.25
Nitrates mg/l		30	28	30	25
Nitrites mg/l		0.34	0.30	0.38	0.10
Phosphates	Ortho mg/l	5	6	5	4
	Meta	3	2	3	3
	Total	8	8	8	7
D.O. mg/l		16.0	15.0	16.0	16.0
H_2S		0.1	0.0	0.0	0.0
Detergent		0.0	0.0	0.0	0.1

LITERATURE CITED

1. Holley, K. R. and E. P. Kane, Resevoir Project Reauthorization: Examples of Past Use and Analysis of Application to Lake Lanier, Georgia Inst. of Tech. Envir. Ctr. ERC-1374, 54, 1974.

2. Strange, J. R., Utilization of Phosphorus by Phytoplankton in Phosporous-Rich Environments, Georgia Inst. of Tech. Envir. Ctr. ERC-1373, 34, 1973.

3. Cook, W. L., D. G. Ahearn, D. J. Reinhardt, and R. J. Reibler, Blooms of an Algophorous Amoeba Associated with Anabaena in a Fresh Water Lake, Water Air and Soil Pollution 3:71, 1974.

4. Ahearn, D. J., E. T. Hall, Jr., and D. J. Reinhardt, Hypnodinium-Like Algal Blooms in Georgia Lakes, Bioscience 21(3):115-116, 1971.

5. Clesceri, N. L. and G. F. Lee, Hydrolysis of Condensed Phosphates--I: Non-Sterile Environment, Intl. Jour., Air and Water Pollut. 9:723, 1965.

6. Jorgensen, S. E., O. Libor, K. Barkacs, Investigation of Phosphorous Removal from Water, Water Res. 7(12):1885-1897, 1973.

7. Kalesy, L., Role of Algae in Sewage Purification: II, Nutrient Removal , Pol Arch. Hydrobio., 20(3) 413-434, 1973.

8. Greer, D. E., and C. D. Ziebell, Biological Removal of from Water, J. Water Pollut. Con. Fed., 44(12):2342-2347, 1972.

9. Peterson, S. A., W. L. Smith, and K. W. Malueg, Full Scale Harvest of Aquatic Plants: Nutrient Removal from a Eutrophic Lake, J. Water Pollut. Con. Fed., 46(4):697-707, 1974.

10. Jones, H., Treatment in Municipal Plants: Innovations for Removal of Phosphorous, Water Res., 7(1/2):211-226, 1973.

11. Stumm, W., Significance of Phosphorus in Lakes and Coastal Water Sediments and Benthos, Water Res., 7(1/2):129, 1973.

12. Odum, E. P., E. J. Kuenzler, and M. X. Blunt, Uptake of P-32 and Primary Productivity in Marine Benthic Algae, Limnol. and Oceanog., 3:340-345, 1958.

13. Keenan, J. D., M. T. Auer, The Influence of Phosphorus Luxury Uptake on Algal Bioassays, J. Water Pollut. Con. Fed., 46(3):532-542, 1974.

14. Schelske, C. L., E. D. Rothman, E. F. Stoermer, and M. A. Santiagoe, Responses of Phosphorus Limited Lake Michigan Phytoplankton to Factorial Enrichment with Nitrogen and Phosphorus, Limnol. and Oceanog., 19(3): 409-419, 1974.

15. Condit, R. J., Phosphorus and Algal Growth in the Spokane River, Northwest Sci., 46 (3): 177-189, 1973.

16. Finenko, Z. Z. and D. K. Krupatkina-Akinina, Effect of Inorganic Phosphorus on the Growth Rate of Diatoms, Mar. Biol., 26(3): 193-201, 1974.

17. Strange, J. R., G. D. Williamson, D. J. Fletcher, P-32 Uptake in Lentic Algae, Environ. Let., 9(1):1-6, 1975.

18. Strange, J. R., J. W. Dean, G. K. Dorman, and D. J. Fletcher, New Method for Recording Heart and Respiratory Rates in Catfish, Sci. of Biol., 1(1): 17-18, 1975.

19. Cairns, J. Jr., J. W. Hall, E. L. Morgan, R. E. Sparks, W. T. Waller,and G. F. Westlake, The Development of an Automated Biological Monitoring System for Water Quality, Va. Poly. Inst., Bull. 59, 50, 1973.

20. Foster, N. R., J. Cairns, Jr., and R. L. Kaesler, The Flagfish, Jordanella floridae, as a Laboratory Animal for Behavioral Bioassay Studies, Proceeding of the Academy of Natural Sciences of Philadelphia, 121 (5): 129-152, 1969.

21. Scheier, A. and J. Cairns, Jr., An Apparatus for Estimating the Effects of Toxicants on the Critical Flicker Frequency Response of the Bluegill Sunfish, Purdue University Engineering Extension Series No. 132, Part 2, 849-855, 1968.

22. Cairns, J., Jr., and R. E. Sparks, The use of Bluegills to Detect Zinc, Va. Poly. Inst., Water Pollut. Control Series, 18050, EDQ, 45, 1971.

23. Sparks, R. E., W. F. Waller, and J. Cairns, Jr., Effect of Shelters on the Resistance of Dominant and Submissive Bluegills (Lepomis Macrochirus) to a Lethal Concentration of Zinc, Bd. of Can., 29(9): 1356-1357, 1972.

24. Sparks, R. E., J. Cairns, Jr., R. A. McNabl, and G. Sutter, Monitoring Zinc Concentrations in Water Using the Respiratory Response of Bluegills (Lepomis Macrochirus Rafinesque), Hydrobiologia, 40(3): 361-369, 1972.

25. Cairns, J., Jr., J. W. Hall, E. L. Morgan, R. E. Sparks, W. T. Waller, and G. F. Westlake, The Development of an Automated Biological Monitoring System for Water Quality, Va. Poly. Inst., Bull. 59, 50, 1973.

26. Cairns, J., Jr., R. E. Sparks, and W. F. Waller, The Relationship Between continuous Biological Monitoring and Water Quality Standards for Chronic Exposure, Bioassay Techniques and Environment Chemistry, Ann Arbor Science Publishers, Inc., pp. 383-402, 1973.

27. Cairns, J., Jr., and W. T. Waller, The Use of Fish Movement Patterns to Monitor Zinc, Va. Poly. Inst., Water Pollut. Control Series, 18050 EDQ, 45, 1971.

28. Rommel, S. A., Jr., A Simple Method for Recording Fish Heart and Operculum Besats Without the use of Implanted Electrodes, Jour. Fish. Res. Bd. of Can., 30(5): 693-694, 1973.

29. Kilambi, R. V., J. Noble, and C. E. Hoffman, Influence of Temperature and Photoperiod on Growth, Food Consumption, and Food Conversion Efficiency of Channel Catfish, Presented at Southern Division American Fisheries Society meeting, Atlanta, Georgia, Sept. 28-30, 1970.

30. Lee, G. F., Role of Phosphorus in Eutrophication and Diffuse Source Control, Water Res., 7(1/2): 111-128, 1973.

31. American Public Health Association: Standard Methods for the Examination of Water and Waste Water, American Public Health Assoc., Inc., New York, 13th ed. 524, 1971.

32. Hill, R. W., Comparative Physiology of Animals, 15-16, Harper and Row, New York, 1976.

33. Hochachka, P. W., Lactate Dehydrogenase in Poikilotherms: Definition of a Complex Isozyme System, Comp. Biochem, Physiol., 18, 261-269, 1966.

34. Morrison, W. J. and Wright J. E., Genetic Analysis of Three Lactate Dehydrogenase Systems in Trout: Evidence for Linkage of Genes Coding Subunits A and B, Jour. Exptl. Zool., 163, 259-270, 1966.

35. Market, C. L. and Faulhaber, I., Lactate Dehydrogenase Isozyme Patterns of Fish, Jour. Exptl. Zool., 159, 319-332, 1965.

GEORGIA WATER RESOURCES RESEARCH PROGRAM

Georgia Institute of Technology
Atlanta, Georgia 30332

Joseph M. Pettit, President

Thomas E. Stelson, Vice President for Research

University of Georgia
Athens, Georgia 30602

Fred C. Davison, President

William L. Hays, Vice President for Instruction

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Georgia Institute of Technology
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Mail inquiries to:

Director, Institute of Natural Resources
University of Georgia
Athens, Georgia 30602

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